THE CONSTRUCTION AND ANALYSIS OF VECTORS BASED ON BOVINE PAPILLOMA VIRUS

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ABSTRACT

The introduction of linearised Bovine papilloma virus I DNA into mouse C127 cells by either calcium phosphate precipitation or microinjection is described. The analyses performed showed that this isolate of BPV-1 recircularised upon introduction into C127 cells and that it replicated as an intact monomeric, extrachromosomal circular form in the resulting transformants. However, these cells also contained a mixture of complex high molecular weight forms most of which were converted to a linear form of approximately BPV-1 size upon digestion with an enzyme which cuts once within the BPV-1 genome. Further analyses of one of the precipitation-derived cell lines revealed that these high molecular weight forms consisted of two components; one was composed of discrete sized concatomeric open and supercoiled extrachromosomal circular forms of up to ~80kb (10 linked BPV-1 genomes) in size. The second component was material detected on agarose gels as a high molecular weight heterogeneous diffuse smear of a size greater than 30kb. A thorough analysis of this diffuse smear revealed that it represented linear forms. Chromosome fractionation suggested that although some of it may represent broken large circular forms, the majority was tightly associated with chromosomes, probably by integration.

Since the BPV-1 isolate behaved similarly to those reported by others, attempts were made to utilise it as a vector, which could be shuttled between transformed mouse cells and E.coli. At first, BPV-1 was inserted into a plasmid pBR328/TK, containing the HSV-TK gene. This failed to replicate as an intact episome in focus selected C127 transformants. Instead they were found to contain BPV-1 sized deleted extrachromosomal forms with little or no plasmid or HSV-TK sequence remaining. HAT selected L TK$^-$ transformants contained only a high molecular weight smear.

Less complex vectors consisting of BPV-1 DNA inserted into various plasmids pBR328, pAT153 and deletion derivatives were constructed to investigate inhibitory plasmid sequences. These were introduced into C127 cells with selection for foci. None of the resulting transformants were found to contain intact, monomeric extrachromosomal forms. In most cases, the plasmid sequences were deleted leaving a BPV-1 sized extrachromosomal form with higher molecular weight structures also present. Occasionally, in undigested samples, only a high molecular weight smear was detected in cell lines.

In order to test that the relative positioning of the BPV-1 enhancer and promoter regions was an important factor, the BPV-1 enhancer was duplicated at either end of the viral insert. When introduced into C127 cells these vectors were found to be more stable, since transformants contained intact monomeric circular vector

DNA which was recoverable into *E.coli*.

Many of the malfunctioning vectors constructed in the course of this work were similar to successful vectors constructed by others. It was thought that the BPV-1 isolate, cell culture conditions or transformation systems utilised could be causing this discrepancy. To test this, various vectors were acquired from other sources and introduced into mouse cells by either calcium phosphate precipitation or microinjection. The results obtained with different vectors were variable, some functioned in the published manner, some functioned only when microinjected and others did not function when introduced by either method. The results suggested that microinjection is a better method for introducing a vector into cells, since it avoids many of the complex rearrangements experienced with the precipitation technique.

ABBREVIATIONS

Amp	Ampicillin
APRT	Adenine Phosphoribosyl Transferase
ATP	Deoxyadenosine 5'-Triphosphate
bp	Base pair
BPV	Bovine Papilloma Virus
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
Cm	Chloramphenicol
^{o}C	Degrees Centigrade
Ci	Curie
Cl	Clone
CIAP	Calf Intestine Alkaline Phosphatase
cpm	Counts per minute
CsCl	Caesium Chloride
dATP	Deoxyadenosine 5' triphosphate
dCTP	Deoxycytosine 5' triphosphate
dGTP	Deoxyguanine 5' triphosphate
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dTTP	Deoxythymidine 5' triphosphate
dpm	Disintegrations per minute
EDTA	Ethylene diaminetetra-acetic acid
EtBr	Ethidium Bromide
GPT	Guanine phosphoribosyl transferase
HAT	Selective medium containing Hypoxanthine, Aminopterin and thymidine

Hepes	N-2 Hydroxyethylpiperazine-N'-2 ethane sulfonic acid
hGH	Human growth hormone
HPRT	Hypoxanthine guanine phosphoribosyl transferase
HSV	Herpes Simplex virus
Ij	Microinjected
kan	Kanamycin
kb	Kilo base pairs
krpm	1000 revolutions per minutes
mA	milliamp's
M-MuLV	Moloney murine leukaemia virus
MPA	Mycophenolic Acid
MT	Metallothionein
NaPPi	Sodium PyroPhosphate
Neo	Neomycin
OAc	Acetate
OD	Optical density
PBS	Dulbecco's Phosphate Buffered Saline
PMS	Plasmid maintenance sequence
rATP	Adenosine 5' triphosphate
rGH	Rat growth hormone
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
SV40	Simian Virus 40
TCA	Tri Chloro Acetic Acid
TE	10mM Tris-HCl pH7.5 1mM EDTA

TEMED	NNN'N'-tetramethyl-1, 2-diaminoethane
Tet	Tetracycline
TK	Thymidine Kinase
Tn	Transposon
Tris	Tris (Hydroxymethyl) aminomethane
tRNA	Transfer ribonucleic acid
UV	Ultraviolet light
V	volts
XGPRT	Xanthine guanine phosphoribosyl transferase
XMP	Xanthine monophosphate
W	Microwatts

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CHAPTER 1

INTRODUCTION

1.1. Introduction of Foreign DNA into Cultured Mammalian Cells

The introduction of foreign DNA sequences into mammalian cells has proved to be a powerful technique for studying the structure and function of eukaryotic genes. The earliest of such experiments relied on the infectivity of various animal viruses such as Human Adenovirus 5 (Graham and van der Eb, 1973) for transforming cultured mammalian cells using naked viral DNA introduced in a calcium-phosphate precipitate. The first gene introduced reproducibly into mammalian cells by the calcium phosphate precipitation technique was the thymidine kinase (TK) gene of Herpes simplex virus (HSV), which was shown to rescue TK deficient cells in HAT selective medium (Wigler et al, 1977; Maitland and McDougall, 1977; Bacchetti and Graham, 1977). It was also found that the inclusion of carrier DNA (i.e. high molecular weight DNA of eukaryotic source) increased the transformation efficiency, and that total DNA prepared from a TK^+ transformed cell line could rescue other L TK^- cells in a second transformation. Wigler et al (1979a) have since shown that the adenine phosphoribosyl transferase (APRT) gene can be transferred into $APRT^-$ mouse cells using genomic DNA from Chinese hamster, human, mouse or salmon cells. The transfer of an altered dihydrofolate reductase gene (DHFR) which provides the cells with a resistance to the folate analogue methotrexate has also been demonstrated (Wigler et al,

1980; Lewis et al, 1980). Similarly, a HPRT gene (hypoxanthine phosphoribosyl transferase) can be used to rescue HPRT$^-$ cells as demonstrated by Willecke et al, 1979 and Graf et al, 1979.

In these reports, the ability to introduce an exogenous DNA sequence into mammalian cells depended on the availability of selectable markers and mutant cell lines deficient in gene products required in biosynthetic pathways. Therefore, the availability of such mutants limits the number of genes introduced by this method. It has been found that when mixed in excess over a selectable marker in a precipitate non-selectable sequences can be introduced into mammalian cells. Such co-transfer of a non-selectable sequence with a selectable marker (HSV TK) was reported for an esterase-D gene, found to be present in 1/15 TK positive recipient L cells (Warrick et al (1980). Co-transfer of the galactokinase K gene (Gal K) in 1/87 TK$^+$ L cell transformants from Chinese hamster genomic DNA was thought to signify chromosomal linkage of the Gal K and TK loci. However, it was equally likely that the genes became linked during the transformation process and therefore represented co-transfer of unlinked markers (Scangos and Ruddle, 1981).

Wigler et al (1979b) demonstrated conclusively that unlinked, non-selectable DNA sequences could be co-transferred with a selectable marker when present in

excess over the marker. DNA sequences from the rabbit β-globin gene, phage ØX174 and pBR322 were mixed in 1000 fold excess over the HSV TK gene and introduced, via a calcium phosphate precipitate, to L TK^- cells. The co-transferred sequences were present in a high percentage of TK^+ cell lines, with copy numbers up to 100. However, it was found that the non-selectable sequences were only retained by the transformed cells, if they remained plated in selective medium after transformation.

1.2. Structure and organisation of exogenous sequences in transformants

A large amount of effort has been put into resolving the fate of the DNA sequences introduced into mammalian cells by calcium phosphate precipitation. The early work of Pellicer et al (1978) demonstrated that the transferred HSV TK gene was associated with high molecular weight DNA, the restriction patterns of the flanking region DNA differing in independent transformants. Wigler et al (1978) showed that when the HSV TK gene on an 8.3kb HpaI fragment, was used to transform L TK^- cells, 3 of its internal BamHI sites were lost in one transformant and when DNA from this primary transformant containing a single copy of the TK gene, was used to retransform L TK^- cells, further rearrangements were found to occur. These findings

suggested that the introduced DNA undergoes degradation and rearrangement on entering the cell prior to its integration.

Transformed cell lines have been found to fall into two classes: stable and unstable. In the former the transferred gene is maintained even in the absence of selection, whereas in the latter class expression of the transferred gene is gradually lost (Wigler et al, 1979b; Graf et al, 1979; Peterson and McBride, 1980; Lewis et al, 1980). A detailed analysis of three independent L TK^+ clones transformed with a HindIII linearised plasmid (pX TK) containing the HSV TK genes as a 3.4kb BamHI fragment, was carried out by Scangos et al (1981). Initially, the transformants expressed the TK gene unstably, with 10% of cells in each generation losing both the TK^+ phenotype and sequence. After a long period of growth in selective medium, stable populations of cells arose which retained the TK gene in the absence of selection. In agreement with the previous results (Pellicer et al, 1978; Wigler et al, 1978) it was found that the input plasmid in unstable cell lines was reduced in size and linked to high molecular weight DNA to form large molecules which could be precipitated in a Hirt fractionation which selectively precipitates large chromosomal sized molecules but small extra-chromosomal molecules remain in the supernatant (Hirt 1967). These large molecules were termed transgenomes which can contain multiple copies of the viral TK gene,

and more than one transgenome can be present in some transformants. Stable derivatives of these cell lines contained only one transgenome which had integrated into a host chromosome, as evidenced by karyotyping in hybrid derivate lines. Furthermore Robins et al (1981) have found by in situ hybridisation that transferred genes are associated with different host cell chromosomes in different cell lines. Transferred genes in unstable cells lines cannot be assigned to a given chromosome suggesting that they are extrachromosomal entities. The formation of transgenomes must take place early in the transformation process since subclones derived from a colony in the 8-20 cell stage all contain an identical arrangement of the transferred sequences (Perucho et al, 1980b). Scangos and Ruddle (1981) have used centrifugation of mitotic chromosome preparations through sucrose gradients, to test the hypothesis that transgenomes are not chromosomally associated. They found that in preparations from unstable cell lines the transferred sequences migrate not with the chromosomes but as a sub-chromosomal species, confirming the above hypothesis.

Co-transfer experiments suggested that the transgenome is formed by the linking of carrier DNA sequences and the cotransferred DNA with the selected gene. Huttner et al (1979) back selected against TK gene expression with bromodeoxyuridine (BrdU) in L cells which had previously been co-transformed to the TK^+

phenotype with the HSV TK and human β-globin genes. All surviving TK^- cells had lost both the HSV TK and globin sequences. Similar results have been obtained by co-transfer of ØX/174 sequences (Perucho et al, 1980b). This supports the hypothesis that co-transferred sequences become linked to the TK gene during the course of transgenome formation. Indeed Perucho et al (1980a,b) recovered by cloning the chicken TK gene from a transformed cell line and found that the sequences flanking it were derived from donor sequences and not host DNA.

Thus the following events are thought to mediate the formation of a stable transformant after transfected sequences enter the recipent cell lines. The transferred phenotype is initially expressed unstably, the selected gene sequences may become rearranged and then linked into a high molecular weight structure (transgenome) composed of carrier DNA sequences and other copies of the selected gene.

1.3. Role of carrier DNA in transformation

It has been found that the transformation efficiency can be greatly increased by addition of carrier DNA to a tranformation mix containing a given amount of the selectable gene (Graham et al, 1979). Carrier DNA is thought to aid the formation of the

calcium phosphate precipitate, making the DNA more acceptable for entry into the cell. In carrier free transformation, although the final transformation frequency is much lower, the frequency at which stable transformants arise is much higher (Huttner et al, 1981).

Carrier DNA may thus provide some function which allows the transgenome to be maintained in an autonomous state in unstably transformed cell lines. These may include:

1. Origins of replication, which could aid in the maintenance of an autonomous, replicating transgenome.

2. DNA sequences, such as enhancers, which could enhance the frequency of expression from integrated selectable markers.

3. Provision of a chromatin structure favourable for gene expression.

Analysis of transformed cell lines generated by the microinjection of DNA directly into the cell nucleus (Capecchi, 1980; Folger et al, 1982) suggested that indeed origins of replication may be provided by the carrier. They found that a plasmid containing an SV40 origin of replication as well as HSV TK gave 100 fold

more TK^+ transformants than HSV TK and plasmid alone. However, it is now known that the SV40 (Simian Virus 40) origin of replication also contains viral enhancer sequences which increase expression from nearby genes. The enhancer sequences from other viral genomes, Avian leukemia virus (Folger et al, 1982), polyoma virus (Yamaizumi et al, 1983) and Bovine papilloma virus (Campo et al, 1983b) have been found to have a similar effect even in the absence of the viral replication origins.

It now seems most likely that the major role for carrier DNA is to form a 'good' calcium phosphate precipitate which can easily be accepted by the cells. Any other role is probably minimal. In the 'carrier free' TK transformations of Huttner et al, 1981, restriction enzyme analyses revealed that the input DNA was integrated as a multimer at one chromosomal location. Estimates of the sizes of carrier-free transgenomes, by hybridisation, range from 50 to 100kb, contrasting sharply with the estimated sizes (up to 2000kb) of transgenomes formed in the presence of carrier DNA (Perucho et al 1980b). TK^+ transformants selected after microinjection of a HSV TK containing plasmid are comparable to those derived by 'carrier free' precipitation. Folger et al (1982) showed that such TK^+ transformants contain from 1 to 100 copies of the injected plasmid as tandem head to tail arrays, at one or a very few sites in the host chromosomes. Independent

transformants contained the TK plasmid on different chromosomes suggesting that integration was not site specific. When injecting either supercoiled or linear plasmid it was found that the head to tail concatomers of the transforming plasmids were formed by homologous recombination rather than ligation of the free ends of linear molecules.

Results in our laboratory (J.P. Simons, Ph.D. thesis, 1985) suggest that during carrier free calcium phosphate precipitate transformation of cells with pBR328 HSV TK (Fig.2.3), plasmid sequences undergo complex rearrangements resulting from degradation of the transferred sequences and/or intermolecular recombination. Such rearrangements were less complex if the plasmid DNA was microinjected into the nuclei of cells suggesting that little or no nuclease activity resides in the nucleus, the results of Folger et al (1982) support this. Thus in calcium phosphate precipitation experiments degradation probably occurs on transport of the DNA through the cytoplasm of the cell to the nucleus. Such cytoplasmic degradation was also suggested by the results of Luthman and Magnusson (1983).

1.4. Development of dominant selectable markers

The selection of TK^+ transformants by introduction of the HSV TK gene has been useful for

introducing other DNA sequences into TK^- cells. A major limitation of this approach is that it relies upon the complementation of a cell mutation (in this case TK^-) by the transferred DNA and is thus restricted by the availability of mutant mammalian cell types. The TK^+ selection system has resulted in only limited expression from non-inducible genes introduced by co-transformations.

A more useful selection system would allow the introduction of any gene into any cell, irrespective of the availability of cellular mutations in that cell line. Such a solution is offered by the cotransfer of genes with a dominant selectable marker (e.g. Neo or Gpt).

Mulligan et al (1979) showed that SV40 control sequences could be used to promote expression of rabbit β-globin cDNA in the vector pSVGT5-RaβG_1. Mulligan and Berg (1980, 1981) advanced the use of SV40 sequences in vector design by linking them to bacterial genes which then became dominant selectable markers in mammalian cells. Inititally the bacterial gpt (guanine phosphorylbosyl transferase) gene was used in the vector pSV_2-gpt which enables transformants to bypass the block imposed by mycophenolic acid on the convertion of IMP to XMP, when included in the medium (see Fig.2.1). Only gpt transformants can convert Xanthine to XMP by use of the bacterial enzyme activity XGPRT, thereby overcoming the MPA block.

Subsequently, a second pSV_2 based dominant selection system has been developed by Southern and Berg (1982a,b) which utilises the cytotoxic action of a synthetic aminoglycoside drug G418. G418 disrupts the function of 80S ribosomes in eukaryotic cells thereby blocking protein synthesis. Jimenez and Davies (1980) found that the bacterial aminoglycoside phospho-transferases APH(3')II and APH(3')I encoded by bacterial transposons Tn5 and Tn601 respectively could inactivate G418, thus the expression of this gene in mammalian cells might protect against G418. The APH3'II gene (designated neo) was subcloned from Tn5 into pBR322, and was then inserted into the pSV_2 plasmid under the control of the SV40 early promoter, and the appropriate 3' intron and polyadenylation signals (see Fig. 2.9)

Analysis of mouse L TK^- cells transformed to G418 resistance by calcium phosphate precipitation in the absence of carrier DNA by pSV_2-neo revealed that the vector sequences were associated with high molecular weight genomic DNA as seen in pSV_2-gpt MPA resistant cells lines (Mulligan and Berg, 1981). The pSV_2-Neo sequences were present at approximately 1-5 copies per genome which were presumed to be organised as single integrated copies each flanked by host cell DNA. In one case a transformant seemed to contain multiple copies of pSV_2-neo arranged in tandem and integrated at a single site. The pSV_2-neo vector provides a favourable selective system since it is directly

selectable in prokaryotic cells where it confers neomycin and kanamycin resistance and in any mammalian cell where it confers resistance to G418. This enables the recombinant plasmids to be shuttled between E.coli and mammalian cells using the same selectable marker, therefore reducing the size of the vector. Colbere-Garapin et al (1981) simultaneously constructed a vector, pAG60, which directed the expression of the Tn5 ApH3'II (neo) gene in mammalian cells from the HSV-TK gene promoter, this construct also allows the selection of G418 resistant transformants (Fig.2.9).

1.5. Microinjection and Expression of Foreign Genes in Transgenic Mice

Clearly, one of the aims in this field of work is to be able to introduce foreign genes into living mammals, in such a way as to obtain developmentally regulated tissue specific expression of those genes, the ultimate hope being that such technology may allow the correction of genetic defects in animals, and eventually humans, by introducing a correctly functioning gene into that animal. Such technology would also have important commercial applications in the manipulation of farm animals to produce large quantities of valuable proteins.

The work described so far has involved the selection of stably transformed cells in culture, but such systems

are highly artificial and only serve as a model for events leading to the expression of a gene *in vivo*. Recent techniques have allowed the introduction of foreign genes into single celled mouse embryos (fertilised oocytes) by direct injection through a glass micropipette. Some of the offspring that develop from these injected embryos have acquired integrated copies of the foreign DNA in the genome of somatic tissues, such mice have been termed transgenic (Gordon and Ruddle, 1981).

The first report of DNA sequences introduced into mice by this method involved a recombinant plasmid which carried the SV40 origin of replication and the HSV TK gene (Gordon *et al*, 1980). Unfortunately, in the analysis of the two putative transgenic mice it is likely that one DNA sample was contaminated with exogenous plasmid DNA which put these results into question. Since then many genes have been introduced into mice by this technique including the rabbit β-globin gene (Costantini and Lacy, 1981; Wagner *et al*, 1981 and Lacy *et al*, 1983), the human β-globin and HSV-TK genes (Wagner *et al*, 1981; Stewart *et al*, 1982), the chicken transferrin gene (McKnight *et al*, 1983), and various gene coding sequences linked to the 5' control region of the mouse metallothionein-1 (MT) gene (HSV-TK, Brinster *et al*, 1981; Palmiter *et al*, 1982a, and the rat or human growth hormone, rGH or hGH, coding sequence, Palmiter *et al*, 1982b; 1983). Usually

between 3 to 40% of the embryos injected result in the formation of transgenic mice and often the exogenous gene could be transmitted through the germ line to the next generation. Frequently it has been found that the foreign sequences are transmitted in Mendelian fashion confirming that they are associated with a host chromosome by integration and thus it has been possible to establish permanent transgenic mouse lines which carry the gene of interest stably integrated into the mouse genome.

In many of these papers the expression of the introduced genes has been examined, and apart from those cases where artificially constructed inducible fusion genes (such at MT-TK, MT-rGH or MT-hGH) have been utilised expression has been aberrant or completely absent. For example, Lacy et al (1983) found that the introduced rabbit β-globin gene had integrated at different chromosomal sites in the five transgenic mice analysed. No expression of the rabbit β-globin gene was detected in erythroid cells of any of these mice, however β-globin gene transcripts were detected in two non-erythroid tissues (i.e. muscle and testis). Similarly, Palmiter et al (1983) found that although expression of the MT-hGH fusion gene in general followed the normal pattern of expression of the endogenous mouse metallothionien-1 gene, in eight tissues of four transgenic mice, occasionally the foreign MT-hGH gene was expressed at a very high level in a tissue where MT-1 expression is normally very low. Harbers et al (1981)

reported that a mouse retroviral DNA resulting from the injection of a cloned form of Moloney Murine Leukaemia Virus (M-MuLV) into oocytes resulted in the integration of a viral genome in the host chromosomes of several transgenic mice. In one mouse this integrated viral genome was expressed at high level in the muscle which is not a lymphatic tissue (i.e. where M-MuLV is normally expressed). These and other reports of aberrant expression of introduced DNA sequences, viral or otherwise, all suggest that the chromosomal position at which the DNA integrates influences the expression of that sequence during development and differentiation. Such position effects probably involve tissue specific alterations in chromatin structure which spreads from expressed host genes into the integrated exogenous genes. It is also conceivable that the integrated foreign DNA sequence could have similar effects on flanking host genes.

The correct timing of expression of introduced genes in the correct tissue must be achieved if such techniques are to be applied to the correction of genetic lesions in humans (gene therapy) or to the engineering of farm animals for, for example, the large scale production of commercially valuable proteins.

Recently, Brinster et al (1983) have provided evidence suggesting transgenic mice derived from embryos injected with a rearranged κ immunoglobulin light

chain gene expressed this gene in a tissue specific manner in the spleen, however, only one other tissue was examined (liver) where no expression of this gene was detected. Since then many tissues have been examined from these mice in which no expression of the κ gene was found (Storb et al, 1984). It is suggested that this controlled tissue specific expression may result from the inclusion of cis acting tissue specific enhancer type sequence on the injected DNA fragment, (similar to those invoked by the results of Walker et al (1983) for cell specific expression from chymotrypsin and insulin 5' control regions), which can induce a chromosomal environment favourable for its expression in the spleen (B. Hogan, 1983). How such cis acting tissue specific enhancer sequences function is not as yet clear, but it is possible that other tissue specific cellular factors are involved in the recognition of enhancer sequences as suggested by Scholer and Gruss (1983).

1.6. Integration of exogenous DNA into functional regions can cause mutation

In general, it has been found that the introduction of exogenous DNA sequences, viral or otherwise, into cultured mammalian cells, by calcium phosphate precipitate or microinjection, or into mouse embryos by microinjection or infection, results in the integration of one or many copies of that DNA into the host genomic

DNA. Clearly, random integration of exogenous DNA into a host genome could result in the inactivation of cellular genes at the site of integration by insertion mutagenesis. For cells transformed in culture, such mutagenic events would be difficult to detect, since selection is for the ability of transformed cells to grow in a selective medium and any cell carrying and expressing a deleterious mutant would be lost. In fact it is unlikely that an integration event would occur in both copies of a cellular gene, so a heterozygous state would be created by integration into one copy, and the deleterious effects of this integration would be masked by the normal homologue of the gene.

The integration of foreign DNA into cellular genes of mouse embryos could result in defects in the developing embryo, although in most cases such insertion mutations will again be masked by the normal allele in the heterozygous state. However, Jenkins et al (1981) have shown that a spontaneous mouse coat colour mutation at the dilute (d) locus is associated with the site of integration of a Moloney Murine Lukemia Virus (M-MuLV) genome since in revertant mice the viral genome has been lost from that locus. Similarly, Copeland et al (1983a) have demonstrated that a dominant mutant allele of the Agouti coat colour locus, A^{y}, may be caused by integration of a proviral M-MuLV at that locus. Homozygous A^{y} mice die on the 6th day of gestation,

but excision of this viral genome leads to reversion of the mutation (Copeland et al, 1983b). Thus it was realised that the insertion of foreign DNA into a cellular gene might be used to identify the function of that gene. Similar insertional mutants have been formed in yeast (Roeder and Fink, 1980 and Roeder et al, 1980) and in Drosophila (Spradling and Rubin, 1981; McGinnis et al, 1983; O'Hare and Rubin, 1983) where insertion of endogenous transposable elements and insertion elements is associated with DNA arrangements and gene mutation and have proved to be invaluable in determining the function of the affected gene.

Jaenisch et al (1983) have tested the possibility that the integration of exogenously introduced M-MuLV at one of their 13 Mov loci (13 independently derived transgenic strains with a copy of M-MuLV integrated at a different chromosomal locus in each strain (see Jaenisch et al, 1981, for details) may have resulted in a recessive insertion mutation. To reveal such recessive mutations they crossed transgenic mice heterozygous for a particular Mov locus and analysed the offspring to identify those which were homozygous at that Mov locus. Mice which were homozygous at any one of 12 of the Mov loci developed normally. However, homozygosity at the Mov-13 locus resulted in the death of the animal early in embryonic development i.e. day 13/14 of gestation. Further analyses of these Mov-13 mice revealed that the viral genome had inserted into the 5' end of the $\alpha 1(I)$

collagen gene (Schneike et al, 1983), suggesting that this viral integration resulted in a complete transcriptional block of the collagen gene, which is normally activated at day 12 of embryonic development. Harbers et al (1984) have since shown that the M-MuLV genome was inserted into the first intron of the α1(I) collagen gene, 19bp down from an intron/exon junction.

Recently Wagner et al (1983) have reported another case of insertional mutation after the microinjection of a plasmid pBR322 containing the human growth hormone gene sequences. Six transgenic mice (30% of survivors) contained the foreign sequences integrated into the host genome in a heterozygous state. These sequences were transmitted to progeny giving rise to 6 new strains (HUGH/1-HUGH/6). Each strain had a unique pattern of integration of the donor sequences which were present as one to 20 tandemly arranged copies. Each of these F_1 HUGH strains was subsequently mated with itself in an attempt to construct strains of mice homozygous at each HUGH locus in the F_2. Blot hybridisation data revealed the existence of healthy homozygotes among the progeny from such matings within four of the strains (HUGH/1,2,5 and 6). In two strains, however, (HUGH/3 and 4) no postnatal homozygotes could be detected and all litter sizes were small, although normal numbers of embryos were present in early development. Matings were also set up between strains HUGH/3 and HUGH/4,

individuals which were heterozygous at both loci were found to be viable and normal. Thus these mutations were due to the integration of the human growth hormone gene into two different essential host genes. The function of the disrupted DNA sequences is, in this case, not as yet known.

Finally, Palmiter et al (1984) have reported an unusal case, where the transmission of inserted sequences does not occur from transgenic males to their offspring but only occurs through transgenic females of that strain. This strain, MAK103, was derived from an egg injected with a plasmid carrying the metallothionein-TK fusion gene (pMK). The embryo developed into a female and contained two copies of the injected DNA present as an inverted repeat. These inserted sequences were transmitted in a normal fashion by females of this strain in the five generations so far examined. Males on the other hand, although carrying the insertion, and being fertile, did not give rise to any progeny carrying the insertion when crossed to a wild type mouse. One possibility is that the integration of foreign DNA disrupted a gene required at some time during the haploid stage of spermatogenesis.

From the above reports, it is clear that the introduction of foreign DNA sequences into an early embryo can result in integration of those sequences into the host genome without any expression or the

desired tissue specific expression, but with the frequent generation of homozygous recessive lethal mutations. Such mutants will be extremely useful in identifying developmentally important genes and for this purpose insertional mutagenesis provides a powerful tool. However, the disruption of functional host sequences would not be desirable in a case where one was trying to correct a genetic lesion by introducing a normal allele of that gene.

In principle there are two ways of avoiding the disruption of native gene functions by the intregration of exogenous sequences. The first is to target these sequences into a part of the genome containing no functional genes. However, site specific integration (targeting) remains to be demonstrated in either cell culture or embryo injection experiments (Berg et al, 1983; J. P. Simons, 1985). In addition, expression of integrated DNA at any chromosomal site cannot, as yet, be guaranteed and it is not known if disruption of such 'gene barren' regions would affect other chromosomal or cellular functions. The second method is to develop a system which allows genes to be introduced without chromosomal integration.

1.7. Extrachromosomal maintainance as an alternative to integration

Such a procedure for introducing donor genes into a host cell without disruption of native DNA sequences would involve the development of a vector which replicates extrachromosomally, thus avoiding any mutagenic integration events. Such a vector would be advantageous in that expresssion of the donor genes would be unhindered by chromosomal position effects or the rearrangements frequently associated with integration. As seen above, the expression from integrated gene copies in either cell culture or transgenic mice has been disappointing. The most encouraging reports being the suggestion from the work of Brinster *et al* (1983) and Storb *et al*, (1984) that the inclusion of a specific *cis* acting control element, with the donor gene, may result in its tissue specific expression in the resulting transgeneic mice, independent of its chromosomal position. This may also be true of the chicken transferrin gene (McKnight *et al*, 1983).

It is, therefore, desirable to have a vector containing a selectable marker which replicates as an autonomous extrachromosomal molecule in transformed cell lines. The SVGT5-RaβG_1 recombinant mentioned earlier replicated in monkey CV_1 cells, with the aid of helper virus, but productively infected the cells resulting in the release of SV40 virions and cell lysis

(Mulligan et al, 1979) which is undesirable if stable, long term expression of a gene is required.

Tsui et al (1982), demonstrated that the pSV_2-gpt vector, which retains the SV40 viral replication origin, can persist extrachromosomally in COS1 cells (Gluzman, 1981), a monkey CV-1 cell line which contains a single integrated copy of the complete early region of SV40 DNA giving endogenous production of SV40 large T antigen, required for the autonomous replication of SV40. The amount of supercoiled pSV_2-gpt in such transformants was estimated to be between 5-2000 copies per cell, most transformants contained intact pSV_2-gpt molecules, although some recovered plasmid molecules contained deletion of pBR322 sequences. It was consistently found however that a proportion of pSV_2-gpt sequences resided in high molecular weight DNA structures which were probably concatemers of pSV_2-gpt and may have been integrated.

Smith et al (1982 - see Southern & Berg) introduced the pSV_2-neo vector into COS cells and found that the G418 resistant transformants contain 10-100 copies of autonomously replicating pSV_2-neo DNA (whether they also contain high molecular weight pSV_2-neo DNA is not known). These extrachromosomal pSV_2-neo molecules have been rescued and shuttled backed into E.coli. The COS cell system is limited in that only artificially constructed COS cells can be used to

induce the stable extrachromosomal replication of molecules containing SV40 replication origins and therefore the range of host cell lines is again limited.

Vectors have been engineered, based on pSV_2-gpt and pSV_2-neo which also encode the viral T antigen (Mulligan and Berg, 1981; Southern and Berg, 1982). These vectors, pSV_3-gpt and pSV_3-neo will only replicate transiently in mouse LTK^- or 3T6 cells. On selection of stable MPA or G418 resistant transformants, vector sequences were found to integrate into the host genome with loss of ability to produce T antigen. Similar results were found using pSV_5-gpt and pSV_5-neo, which contain the polyoma virus (a mouse virus) T antigen gene and replication origin. Therefore some other system is required to achieve expression from an episomal molecule in mammalian cells.

The work presented here is concerned with the development of an extrachromosomal replicating vector which, it is hoped, will eventually allow any foreign DNA sequence to be maintained and expressed extrachromosomally in mammalian cells. The most promising starting point for the construction of such vectors seems to be Bovine Papilloma Virus Type-1(BPV-1), a member of the papovavirus family.

1.8. Bovine Papilloma Virus type 1 (BPV-1) - extrachromosomal replication in transformed cells

The papilloma viruses are small DNA viruses, which induce squamous epithelial tumors in many higher vertebrates including man. As yet no papilloma virus has been successfully propagated in cell culture, possibly because the functions required for productive growth are only expressed in differentiating squamous epithelial cells (Howley, 1983). As a result of the lack of an *in vitro* system for propagating BPV, the molecular genetics, and therefore an understanding of the functional regions, of the BPV genome have been difficult to investigate and this has hindered its development and use as a mammalian cell vector.

BPV type 1 (BPV-1) has been the most extensively studied papilloma virus. It induces fibropapillomas in its natural host (cattle), however, it can also induce fibromas in hamsters and mice (see Howley, 1983 for references) and *in vitro* transformation of foetal bovine conjunctiva, embryonic calf skin, embryonic mouse and embryonic hamster cells in culture has been demonstrated (Black *et al*, 1963, Boiron *et al*, 1964, Thomas *et al*, 1964 and Geraldes, A. 1969). DNA from BPV-1 virions was found to transform rodent cells in culture and induce tumours in hamsters (Boiron *et al*, 1965). An assay system has been developed to identify BPV-1 transformants in cultured non-transformed

fibroblast cell lines (Dvoretzky et al, 1980). This system clearly identifies a transformed colony or focus which results in the appearance of a pile of non contact inhibited transformed cells on a monolayer background of non-transformed, contact-inhibited cells. These transformed mouse cells do not contain infectious virus particles, that is they are non-permissive to viral production, but form tumors when placed in nude mice. This focus selection system allows quantitative analyses to be performed on BPV-1 transformations and the isolation of independently BPV-1 transformed cell lines.

BPV-1 DNA has been cloned in pBR322 and progagated in E.coli. When separated from the plasmid by digestion with a restriction enzyme, the resulting BPV-1 DNA retains its ability to transform cultured mouse NIH3T3 or C127 cells (Lowy et al, 1980). It has also been shown that a HindIII/BamHI subgenomic fragment containing only 69% of the BPV-1 genome (BPV 69%T) also retained this transforming activity. However, from this report it is not clear whether it is necessary to separate the plasmid sequences from the BPV-1 genome in order to obtain efficient transformation. It was later demonstrated (Law et al, 1981) that cell lines derived from foci induced either by the BPV-1 virions, the complete BPV-1 genome which had been released as a linear fragment by digestion with HindIII or BamHI or the linear 69% subgenomic fragment (BPV69%T), all contained the BPV-1 sequences as multicopy extrachromosomal supercoiled and

open circular forms. Only monomeric forms of the viral DNA were found in cell lines transformed with BPV-1 virus particles, but, in those transformed with the linear forms derived from cloned BPV-1 DNA there was a large proportion of high molecular weight material in addition to the monomeric circular forms detected by blot hybridisation experiments of undigested DNA samples. Restriction enzyme analysis suggested that all these high molecular weight forms represented multimeric extrachromosomal forms of the viral DNA, since all the viral DNA sequences could be converted to a single band the size of linear BPV-1 (8kb) with an enzyme which cuts only once per genome. No junction fragments indicative of integration could be detected. Similar analysis of BPV69%T transformants suggested that the introduced viral fragment underwent complex rearrangements, perhaps involving duplication and deletion, on entering the cell which resulted in replicating forms of a size larger than the input DNA. It is possible that the larger size of these molecules resulted from the incorporation of host cell or carrier DNA into the introduced BPV69%T fragment.

Law et al (1981) ruled out integration of viral sequences into the host chromosomal DNA on the basis of digestions of their transformant DNAs with single cut enzymes and comparison of these with reconstruction experiments where 0.1 copies of BPV per cell could be detected. They have argued that an unusual band generated from a host cell/integrated viral DNA junction, would

have been detected, but no such bands were detected. Since mild S1-nuclease treatment resulted in monomeric sized BPV-1 forms it was concluded that none of the high molecular weight molecules of viral DNA detected in transformed lines represented integrated BPV-1, but that these were composed entirely of catenated (interlocked circular) forms. Despite these results the structure of the high molecular weight form of viral DNA and whether or not a proportion of it is integrated into the host genome has not been investigated thoroughly. Since a major part of this thesis is concerned with the physical state of introduced BPV-1 DNA this will be discussed in detail later (see Chapter 7). It is worth mentioning, however, that there are two other reports which also show extrachromosomal BPV-1 genomes as monomeric open and supercoiled circles in bovine tumours and in virus transformed cells (Lancaster et al, 1981) or in BPV-1 induced hamster fibromas and fibrosarcomas (Pfister et al, 1981).

From these results it would appear that BPV-1 viral DNA can transform certain cultured cell lines and induce tumours in vivo. The viral DNA replicates in the nucleus (Howley, 1983) where the majority of the it can be detected as an extrachromosomal form with integrated sequences remaining undetected. This suggested that BPV-1 would form the ideal basis for a vector to introduce and express foreign genes in mammalian cells extra-chromosomally as distinct from systems, discussed

previously, which lead to integration.

1.9. Bovine Papilloma Virus type 1 as a vector for mammalian cells

Since the initial reports which claimed that BPV-1 replicates 'exclusively' as an extrachromosomal circular molecule when introduced into cultured cells, many attempts have been made to 'harness' this virus so as to enable other eukaryotic genes to be introduced and expressed from an episomal hybrid molecule. In many cases the results at first looked promising, but on closer examination one can see that the vectors were not functioning in the required manner. Before discussing these vectors at any length some consideration should be given to the most useful properties that a truly extrachromosomal replicating vector should possess:

1. the vector should replicate, solely, as an extrachromosomal monomeric episome in mammalian cells.

2. the vector should be maintained in an unrearranged state once introduced into the cell and should do so even after prolonged passaging of a cell line.

3. the vector DNA should not integrate into the

host genome, such an event would defeat the whole objective.

4. the vector should tolerate the inclusion of a bacterial origin of replication and selectable marker thus allowing its large scale production in E.coli and allowing its recovery from transformed mammalian cells, i.e. it should be a 'shuttle' vector.

5. the inclusion of a marker which is selectable in mammalian cells (such as HSV-TK, dihydrofolate reductase (DHFR), Metallothionein, neo or gpt) could allow its introduction into a wider host cell range and this is a desirable property for reasons discussed earlier. It would also provide an alternative to the rather tedious focus selection system which is required for the selection of BPV-1 transformants.

6. the complete vector should contain useful cloning sites for the easy introduction of other DNA sequences of interest into the vector.

7. the vector must obviously retain the above properties (1-5) even in the presence of other DNA sequences (introduced in 6).

Many of the vectors constructed, to date, satisfy some of these properties, but as yet no vector has been constructed which possesses all of them.

Sarver et al, (1981) first reported the use of BPV as a vector to introduce an exogenous gene into cultured mouse C127 cells. They used a construct consisting of the rat pre-pro-insulin gene (rI_1) linked to BPV69%T fragment, which was initially cloned in the plasmid pBR322 thus allowing its large scale preparation. However, prior to transfection of mammalian cells the bacterial plasmid sequences were cleaved away. The reason for removing the plasmid sequences was unclear but it was later revealed by DiMaio et al (1982) and others, that the linkage of pBR322 to BPV-1 greatly reduced the frequency of detectable transformed foci. On analysis of the vector sequences in any of the rare transformants arising, deletions had occurred within the plasmid sequences in the extrachromosomally replicating forms. Overall the work of Sarver et al (1981) suggests that the BPV-1 system forms the useful basis for an episomal vector, since they obtained extrachromosomal replication of this BPV69%T-rI_1 hybrid molecule. Both monomeric circular and higher molecular weight forms were detected and the correct expression of the rI_1 gene occurred from those molecules, therefore the inclusion and expression of another DNA sequence did not appear to disrupt extrachromosomal replication. As in the case of the original BPV-1 transformation experiments, it was

claimed that all the high molecular weight complex was converted to a linear monomeric sized BPV69%T-rI$_1$ molecule on digestion with one cut enzymes. However, on closer examination other unexpected bands are visible which could represent either integrated copies or rearranged forms. Because, the plasmid sequences were removed from this construct before transfection it was not possible to recover the replicating forms into bacteria, a property which would be desirable.

Subsequent reports have demonstrated that the inhibitory effects of bacterial plasmid sequences on the transformation and replication of BPV-1 containing molecules can be overcome. This was achieved in two ways; firstly, by the inclusion of a mammalian DNA sequence which by some unknown mechanism allowed the vector to replicate as monomeric circular forms with both plasmid and BPV DNA linked (DiMaio et al, 1982, Kushner et al, 1982; Karin et al, 1983) or, secondly, by deleting parts of the bacterial plasmid DNA which were supposedly inhibitory to the replication of the virus linked to the plasmid in mammalian cells (Sarver et al, 1982).

In the former case DiMaio et al (1982) inserted the BPV69%T fragment into a pBR322 derivative called pBRd, giving pBPV/H11 which was only found to transform mouse C127 cells at a very low frequency. pBRd does not contain the bacterial plasmid sequences which were found

to inhibit the replication of SV40-pBR322 hybrids in transfected monkey cells, the so-called SV40 poison sequences (Lusky and Botchan, 1981). However, on insertion of a 7.6kb fragment of DNA containing the human β-globin gene to produce the vector pBPV-β_1 the transformation frequency was increased by about 500 fold. Analysis, by shearing, of total cellular DNA from cell lines transformed with this vector pBPV-β_1, revealed that it was present as multicopy (10-30) unrearranged extrachromosomal monomeric circles. On digestion with a single cut enzyme it was found that each cell line contained a major band which migrates with the linear input plasmid. DNA extracted from these cell lines could transform E.coli to ampicillin resistance, thus demonstrating that the vector could be recovered intact from mammalian cells.

In one of the cell lines transformed with pBPV-β_1 lower molecular weight forms of the vector were detected which were said to represent deleted or rearranged molecules. Apparently the prolonged passage of other cell lines also resulted in the appearance of rearranged forms. It was also suggested that the possibility remained that a small portion of the vector had integrated into the host genome but went undetected. The introduction of a selectable marker (either HSV-TK or gpt) into the pBPV-β_1 vector results in its integration into the host genome (D.DiMaio, pers. commun.). Similar results have been found by Kushner et

al (1982) where a similar pBR322 derivative containing the BPV69%T sequences and the rat growth hormone gene was found to replicate as an intact episome in transformed C127 cells. The introduction of this vector into L TK^- cells by cotransfer with a plasmid bearing the HSV-TK selectable marker resulted in its integration in TK^+ mouse L cell transformants.

As mentioned above the deletion of specific sequences from the bacterial plasmid was also found to enable BPV-1 vectors, while still linked to the remaining plasmid sequences, to replicate in mammalian cells (Sarver et al, 1982). In this case the plasmid sequences used were derived from pML2 (the in vivo deletion derivative of pBR322, capable of allowing replication of linked SV40 in monkey CV-1 cells), and contained the complete BPV-1 genome inserted as a BamHI fragment. In the course of its construction the small 344bp HindIII-BamHI fragment of pML2 was also deleted, the final vector was called pML2d BPV-1. It was found that as in the case of DiMaio et al (1982) and Kushner et al (1982) this vector replicated as an extrachromosomal monomeric circle and could be recovered into E.coli after propagation in mouse C127 cells.

In this report, Sarver et al (1982) also analysed cell lines transformed by a vector which differed from pML2d BPV-1 in that it contained all the pBR322 sequences. In contrast to pML2d BPV-1, it was found that

transformation was inefficient and that in those rare transformants obtained (two in all) the extrachromosomal molecules contained large deletions covering the plasmid DNA. It was therefore concluded, contrary to the results of DiMaio et al, (1982), that the same plasmid sequences found to inhibit SV40 replication in pBR322 recombinants have a similar effect on the replication of BPV-recombinants.

The results of Sarver et al (1982) contrast sharply with those of Binetruy et al (1982a and b) where it was found that BPV-1 cloned in the plasmid pML2 (pMH_4 and pMB_2) gave rise to transformants at a low efficiency by calcium phosphate precipitation in both mouse C127 and rat FR3T3 cells. However, transformants were obtained with these vectors by bacterial protoplast fusion but both vectors underwent large deletions in both cell types, and only a few cell lines contained any intact vector. In the case of pMH_4 the deletions were seen to span the junction between the BPV3**1%NT** region and the adjacent plasmid sequences up to and including the bacterial replication origin. Possible reasons for such conflicting results with what are very similar vectors will be discussed in a later chapter (see Chapters 4 and 5).

Since the original report of the stimulatory effect of the human β-globin gene fragment on plasmid-linked BPV-1 transformation efficiency, the same group modified

this very large vector (16kb) systematically reducing the size of the human β-globin fragment required to retain this property. In so doing they constructed a deletion vector pBPV-BV1, containing only 4kb of the 7.6 HindIII β-globin fragment, which is approximately 12kb in size (Zinn et al, 1983). The human β-interferon gene (β-IFN) gene was then inserted into this vector giving pBVIF, which was introduced into mouse C127 cells and was found to replicate as an unrearranged extrachromosomal circle in many of the transformed cell lines examined. Further analysis revealed that expression of the β-interferon gene from this extrachromosomal vector was inducible with the synthetic double-stranded RNA, poly(I)-poly(C) and the level of expression in independent cell lines was remarkably consistent.

This result contrasted greatly with other attempts to obtain reproducible expression of the β-IFN gene from BPV vectors (Zinn et al, 1982; Mitrani-Rosenbaum et al, 1982) where the levels of inducibility varied over a 10-fold range in independently derived cell lines. Zinn et al (1983), went on to characterise the regions adjacent to the β-IFN gene responsible for its constitutive or induced expression by constructing deletions within the 5' flanking sequences and assaying their activity when introduced into C127 cells. This report demonstrated how potentially useful BPV-1 based vectors might be for systematically investigating the regulated expression of a gene. However, in the light of

the above results, DiMaio et al (1984) unexpectedly found that introduction of a human HLA gene into the pBPV-β_1 vector resulted in that vector (pBPV-βHLA-1 or 3)integrating into the cellular DNA when introduced into mouse C127 cells. This report served to demonstrate that a BPV-1 vector may not be capable of tolerating the introduction of any eukaryotic DNA fragments and/or may have a certain size limitation.

1.10. Introduction of a Selectable Marker into BPV-1 vectors

One desirable property of a BPV-1 vector would be for it to contain a selectable marker which would allow its introduction into other cell types apart from contact-inhibited fibroblast lines, such as C127. There are many markers which have been linked to BPV-1 in the hope that their selection would allow its properties of extra-chromosomal replication to be utilised in other already morphologically transformed cell lines where focus selection cannot be performed. These markers include the HSV-TK gene (Lusky et al, 1982/1983; Sekiguchi et al, 1983; Kushner et al, 1982) the gpt dominant selectable marker (Law et al, 1982) the human metallothionein II gene (Karin et al, 1983) and the DHFR gene (Breathnach, 1984). However, most or all these markers have been found to result in the integration of the vector DNA into the host genome or result in some

other rearrangement of the vector. On the other hand, it has been found that the selection for G418 resistant transformants upon introduction of a vector (pCGBPV) containing the complete BPV-1 genome and the TK-neo hybrid gene (Colbere-Garapin et al, 1981 - see Fig. 2.9) results in $G418^R$ C127 cell lines where the vector replicates as an extrachromosomal, unrearranged, monomeric circle (Matthias et al, 1983). It was found that this vector only functioned when transcription from the TK-neo hybrid gene was in the direction opposite to that of the BPV-1 genome that is the $pCGBPV_9$ orientation rather than $pCGBPV_7$. Similarly, Law et al (1983) and Lusky and Botchan (1984) have constructed BPV-1 vectors containing the G418 resistance marker, but, in these cases the neo coding sequences were promoted by the mouse metallothionein I promoter and the Harvey Sarcoma virus long terminal repeat, respectively.

In all the reports these respective G418 selectable BPV-1 vectors were found to replicate extrachromosomally as monomeric circular forms in G418 resistant C127 cell lines. In many cases restriction endonuclease digestion revealed the presence of additional unexpected bands which were interpreted as representing rearranged vector sequences and high molecular weight forms. In addition to the generation of $G418^R$ transformants with the $pCGBPV_9$ vector, Matthias et al (1983) also selected for focus formation. No results were shown, but it would be expected that in these cell lines some of the vector

sequences would be deleted, since many of these were not resistant to G418 and thus must have lost the neo gene.

Meneguzzi et al (1984) also reported the use of the TK-neo gene as a selectable marker in BPV-1 vectors, however, their vectors were based on a pBR322 plasmid pAG60, containing the BPV69%T fragment. These were introduced into mouse C127 fibroblasts by protoplast fusion or the calcium phosphate method. Unlike the results of Matthias et al (1983), no monomeric episomal forms were detected, with only a high molecular weight form composed of the input vector DNA being detected. Digestion of DNA from these cell lines generated by protoplast fusion with single cut enzymes, produced a major band of size equal to the monomeric linear form of the vector. The authors conclude that the plasmid was present as unrearranged oligomeric molecules in an unintegrated state. They refer to this state as "plasmidial". A close examination of the results reveals unexpected bands indicative of rearranged or integrated forms. Analysis of $G418^R$ cell lines generated by the calcium phosphate precipitation procedure gave similar results. In cell lines selected by focus formation the vector sequences were rearranged and deleted, the deletions mainly affected the plasmid sequences.

It would appear that the precise nature of the plasmid sequences used in a vector determines whether or

not it will replicate as a monomeric circular form when placed in a mammalian cell. Exactly which plasmid sequences should be used is, to say the least, unclear since results are conflicting. Those of Sarver *et al* (1982), Law *et al* (1983) and Lusky and Botchan (1984) would suggest that the pML2 sequences do not inhibit the replication of BPV-1, whereas those of Binetruy (1982), Matthias *et al* (1983) and Meneguzzi *et al* (1983) suggest that such vectors do not replicate in the desired manner. However, the general consensus of opinion would suggest that a vector containing the complete BPV-1 genome together with a *neo* hybrid gene as a selectable marker will replicate as an extrachromosomal circular form in G418 selected cell lines, provided the plasmid sequences are compatible. The *neo* system has a major advantage in that it can also be used to select kanamycin or neomycin resistant colonies in *E.coli* and therefore reduces the size of the bacterial sequences required. Matthias *et al* (1983), Law *et al* (1983) and Lusky and Botchan (1984) have all demonstrated the recovery of their respective plasmids as kanamycin selected colonies on transformation of *E.coli* with DNA extracted from $G418^R$ mammalian cells.

1.11. Mapping functional regions of the BPV-1 genome

The presence of a viral enhancer sequence in the BPV-1 genome was suggested by the results of Lusky *et al*

(1982, 1983) and Campo et al (1983), where it was found that the frequency of TK^+ colonies formed was greatly increased by using various constructs containing BPV-1 or subgenomic fragments of BPV-1 with the HSV TK gene as opposed to those where BPV-1 was absent. Lusky et al (1982, 1983) found (as mentioned above) that the vector sequences in the resulting TK^+ transformants were always associated with high molecular weight DNA. They were probably integrated into the host genome and therefore the increased transformation frequency was not a consequence of autonomous replication of the BPV vector. It was thought that the BPV-1 genome might contain a regulatory element similar to SV40, since it had been shown that a region from the SV40 origin of replication also increased the TK transformation frequency when included in a vector (Cappecchi et al, 1980). This hyopthesis was tested by Lusky et al (1982) who replaced the 72bp repeat sequences of the SV40 genome with subgenomic fragments of BPV-1. The functional region of the BPV-1 genome was identified by its ability to allow the SV40-BPV recombinant to replicate in monkey CV-1 cells. The region of BPV-1 responsible for this activity was mapped to within a 2.9kb BamHI/BglII fragment and then to a 60bp Sau3A/BamHI fragment (Lusky et al, 1983) (see Fig.1.1). The properties of this fragment were found to be consistent with it being an activator or enhancer sequence, and deletion mutants have since been constructed in an attempt to understand its mechanism of function (Weiher and Botchan, 1984).

FIGURE 1.1. Physical map of the BamHI cleaved BPV-1 genome, showing the relative positions of various functionally defined regions.

PMS 1 & PMS 2 : plasmid maintenance sequences, mapped by Lusky and Botchan (1984) and Waldeck et al (1984).

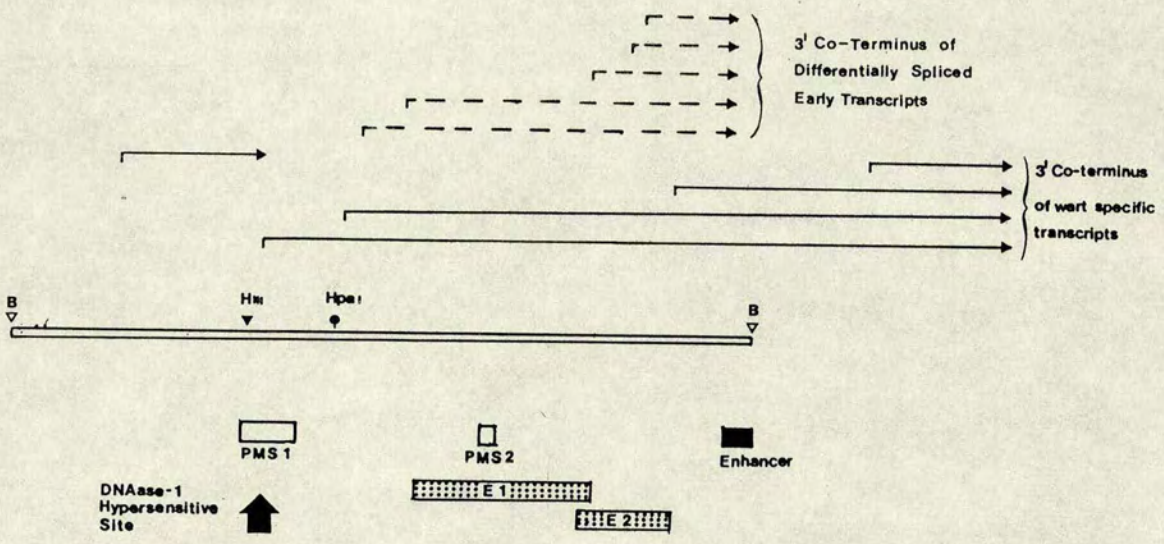
DNAase-1 hypersensitive site indicated by large heavy arrow, mapped by Rosl et al (1983).

Enhancer sequence indicated by dark box was mapped by Campo et al (1983), Lusky et al (1983) and Weiher and Botchan (1984). It resides on a 60bp Sau3A/BamHI fragment.

E 1 : represents the open reading frame thought to encode a protein required for replication (Engel et al, 1983; Clerant and Sief, 1984).

E 2 : represents the open reading frame thought to encode the transformation protein (Engel et al, 1983; Nakabayashi et al 1984; Lusky and Botchan, 1984).

The complete BPV-1 genome has been sequenced by Chen et al (1982.)


3' Co-Terminus of
Differentially Spliced
Early Transcripts
3' Co-terminus
of wart specific
transcripts
B
Hin
Hpa I
B
PMS 1
PMS2
Enhancer
DNAase-1
Hypersensitive
Site
E 1
E 2

The G418 selection system was exploited by Lusky and Botchan (1984) to map the viral sequences required for the maintenance of BPV-1 as an extrachromosomally replicating plasmid (replication origins) in transformed cells and those responsible for the transforming function. Using their pML2 neo vector (i.e. pNeo5';neo structural gene sequences under the control of the promoter from the Harvey Murine Sarcoma virus LTR, in pML2) they tested the ability of subgenomic fragments of BPV-1 to maintain the vector as an episome in either non-transformed C127 or BPV-1 transformed ID13 cells (a C127 cell line previously transformed with BPV-1 virions - Law et al, 1981). It was found that both BPV-1 or BPV69%T or either of two fragments from the BPV-1 genome containing sequences from between positions 6945-7476bp (PMS-1) or 1515-1655bp (PMS-2) on the BPV-1 map, (Fig.1.1) cloned into the pNeo5' vector, allowed the vector to replicate as monomeric episomes in G418 resistant ID13 cells. Only those containing the complete BPV-1 genome or the BPV69%T fragment could replicate episomally in G418 resistant C127 cells. These results suggested that the vectors containing either of the PMS (plasmid maintenance sequence) elements do not produce an essential viral product which is required for episomal maintenanace and that this product was provided in trans by the endogeneous BPV-1 in the transformed ID13 cells. In C127 G418 resistant cell lines transformed by vectors which only contained those BPV-1 subgenomic PMS

elements, the vector DNA was only present in a high molecular weight form and probably integrated into a host chromosome. Further evidence for the extrachromosomal maintenance of the PMS containing vectors in $G418^R$ ID13 cells was provided by the fact that the input plasmid DNA could be rescued from the eukaryotic DNA by transformation of E.coli.

All open reading frames on the BPV-1 genome have been located from BPV-1 DNA sequence data (Chen et al, 1982 and Howley, 1983). To map one of the viral encoded functions, the transforming protein gene, Lusky and Botchan (1984) generated deletion mutants within the region of BPV-1 encoding the 3' end of BPV-1 transcripts, that is the E2 open reading frame (see Fig.1.1). It had previously been reported that gene product(s) of this region were responsible for the transforming function of BPV-1 DNA (Nakabyashi et al, 1983). Lusky and Botchan (1984) found that their two deletion mutants could not produce foci when transfected into C127 cells. When co-transfected with the pNeo5' vector into C127 cells the resulting G418 resistant cell lines contained integrated pNeo5' sequences but the BPV E2 deleted DNA was found to be present as extrachromosomal monomeric circles, even though the cell lines still maintained a non-transformed flat morphology. Indeed these co-transformed cell lines remained susceptable to transformation by vectors containing intact BPV-1 genomes which could be selected for by focus formation. These

results suggest that BPV-1 extrachromosomal maintenance does not depend on the viral products responsible for the transformed phenotype and thus the plasmid maintenance and transformation functions of BPV-1 can be uncoupled, a necessary achievement if such vectors are eventually to be used in whole animal experiments.

One important point concerning the location of PMS-1 is that it is contained within the region of the BPV-1 genome which is postulated to contain the BPV-1 transcriptional regulatory sequences (Chen et al, 1982). A major DNase-hypersensitive site has also been mapped to this region (Rosl et al, 1983, see Fig.1.1). This organisation of functionally important features in the BPV-1 genome is reminiscent of the organisation of the SV40 genome and indeed the replication of PMS containing vectors in ID13 cells is very similar to the situation seen in COS cells, where an endogeneously produced SV40 T antigen allows the replication of vectors containing only the SV40 origin of replication (see Section 1.7). Whether a transacting factor of BPV-1 acts in a similar manner by association with the PMS elements remains to be seen. Recently, Waldeck et al (1984) confirmed, by electron microscopy, that PMS-1 corresponds to the replication origin of BPV-1 in hamster cells.

1.12. Aims of Thesis

There are many reasons for constructing a vector which truly replicates as an unrearranged episomal monomer in transformed cells. It would be advantageous if such a vector also contained the relevant bacterial sequences which would also allow it to be rescued from eukaryotic cells and propagated quickly and efficiently in E.coli. The inclusion of a dominant selectable marker in this vector would greatly increase the host range of the vector and at present the bacterial neo gene linked to a eukaryotic promoter allowing selection of G418 resistant cell lines in any mammalian cell seems the most promising.

This thesis describes experiments which set out to construct BPV-1 containing vectors, based on data available at the time of initiation, which might satisfy some of the properties of the ideal BPV-1 vector set out in Section 1.9. These vectors were then modified according to any new data arising over the period. A second major aim has been to identify those bacterial plasmid sequences which appear to inhibit BPV-1 replication and remove their inhibitory effects either by deleting parts of the plasmid sequences or in some other way manipulating the structure of the vector. The third main aim has been to investigate the oligomeric structures formed in transformed cell lines and to establish as far as is possible whether viral DNA

integrates into the host genome in a BPV-1 transformed C127 cell line. The results of these studies are presented in the following Chapters and are discussed in relation to published literature.

CHAPTER 2

MATERIALS AND METHODS

2.1. Growth and transfection of prokaryotic and eukaryotic cells

2.1.1. Bacterial Culture

2.1.1.A. Bacterial Stains

Escherichia coli strain HB101:

F^-, hsdS20 [r,m], recA13, ara-14, proA2, lacY1, galK2, rpsL20[Sm^r], xyl5, mtl-1, supE44, λ^-.

The strain E.coli HB101 (Boyer and Rouland-Dussoix, 1969; Bolivar and Backman, 1979) was used as the recipient in most transformation experiments and was used as host for large scale growth and purification of plasmids except for isolation of dam^- plasmid forms where CSH26 or GM161 was used (see below).

Escherichia coli strain CSH26:

F^-, SM^R, ara B, (Lac-pro), rpsL dam^- was obtained from N. Murray, Department of Molecular Biology, University of Edinburgh.

Eschericha coli strain GM161:

F^-, thr1, leu6, thi1, supE44, lacY1, tonA21, dam4, hss2(r^-m^-) was obtained from J. O. Bishop, Department of Genetics, University of Edinburgh.

2.1.1.B. Bacterial Culture Media

Strains HB101, CSH26 and GM161 were cultured in liquid L-broth or on L-agar plates.

L-broth medium contains: 1% Difco bacto tryptone
0.5% Difco yeast extract
0.5% NaCl
0.1% Glucose
10mM $MgSO_4$
pH is adjusted to 7.2 with NaOH

L-agar medium contains the above with the addition of 1.5% Difco agar

2.1.1.C. Antibiotics

Ampicillin (Sigma) was made up as a 1000x stock solution in sterile dH_2O at 50mg/ml.

Chloramphenicol (Sigma) was made up as a 34mg/ml stock in 100% ethanol and used at a final concentration of 10µg/ml.

Kanamycin (Gibco) was supplied as a 10mg/ml solution and was used at a final concentration of 50µg/ml.

Tetracycline (Sigma) was made up as a 12.5mg/ml stock solution in 1:1 ethanol:water and stored in the dark. It was used at a final concentration of 15µg/ml.

2.1.1.D. Transformation of Bacterial Cells

The E.coli strain HB101 (Boyer and Rouland-Dussoix, 1969) (see Section 2.1.1.A) was used for growing all plasmids except when deoxyadenosine methylation negative (dam^- plasmids) were required, which were therefore grown in E.coli CSH26 or GM161 dam^- strains (see 2.1.1.A).

Frozen competent cells were prepared by the method of Morrison (1979). A single colony of E.coli HB101 was picked from an L-agar plate and used to innoculate 100mls of L-broth. On reaching an OD_{550} of 0.5 the culture was chilled on ice for 10 minutes. The cells were then spun down at 2,500 rpm for 5 minutes in two 50ml Falcon tubes. The two cell pellets were each resuspended in 12.5mls of ice cold 0.1M $MgCl_2 6H_2O$. The cells were again pelleted and then resuspended in a total volume of 15mls of 0.1M $CaCl_2$ (0^oC). This was left on ice at 0^oC for 20 minutes after which the $CaCl_2$ treated cells were pelleted again and this final cell pellet was resuspended in 4.3mls of 0.1M $CaCl_2$, into which 0.7mls of glycerol were mixed.

These competent cells were then flash frozen (in liquid nitrogen) as 100µl aliquots in sterile eppendorf tubes and stored at -70°C.

The number of cells in each 100µl aliquot is sufficient to carry out one transformation experiment. Before transformation the required number of tubes were thawed on ice for 10-15 minutes, the DNA was then added in a small volume of TE pH7.5 or 1x ligation buffer (usually <10µl/tube). The tubes were kept on ice for an additional 30 minutes and then incubated in a 42°C water bath for 90 seconds.

The transformation mix was then diluted 10 fold, by the addition of 900µl of L-broth, and then incubated at 37°C for 1 hour to allow expression of the plasmid encoded antibiotic resistance genes before growth on selective medium. Normally 50 to 200µl of the incubated mix was plated directly onto L-agar plates containing the appropriate antibiotic. This was spread using a bent sterile (L shaped) Pasteur pipette.

Competent HB101 cells prepared by this method were stored for up to 6 months without any significant change to their transforming ability. For a plasmid such as pAT153 the transformation frequency varied from 2 x 10^5 to 1 x 10^6 colonies per µg. The transformation frequencies obtained with the dam$^-$ strains CSH26 and GM161 tended to be lower.

2.1.2. Mammalian Cell Culture

2.1.2.A. Mammalian Cell Lines and Medium

a) Ltap207:

Ltap207 is a clone (isolated by C. Tyler-Smith, this laboratory) derived from the mouse fibroblast cell line Ltap20 which is adenosine phosphoribosyl transferase negative ($aprt^-$) and thymidine kinase negative (tk^-) and was a gift from F.L. Graham (McMaster University, Canada).

Ltap207 cells were maintained as monolayer cultures at $37^oC/10\%CO_2$ in minimal essential medium, alpha modification (Gibco), supplemented with 10% Donor calf serum (Flow Lab.) 50μM bromo-deoxyuridine (BrdU) and 10u/ml penicillin and streptomycin (Gibco). The inclusion of BrdU selects against TK^- revertants. TK^+ cell lines were selected for and maintained in the same medium except that BrdU was replaced by HAT (Littlefield, 1964). HAT consists of 1μM hypoxanthine, 0.16μM thymidine, 0.004μM aminopterin and .03μM glycine.

In DNA transfection experiments (see section 2.1.2.E) prior to addition of DNA, the Ltap207 cells were maintained on non-selective medium that is as above but with neither the BrdU nor the HAT supplements. Selection for G418 resistant cell lines was applied by the

addition of G418 (Gibco) at a concentrations of 1200µg/ml of non-selective medium.

b) C127:

C127 are a non-transformed mouse fibroblast cell line derived from a mammary tumour of an RIII mouse (Howley et al, 1983). This cell line grows as a flat monolayer at 37°C/10%CO_2 with no overgrowth visible and thus like NIH3T3 cells (Dvoretzky et al, 1980) are susceptible to Bovine papilloma virus (BPV) induced transformation and thus focus selection.

The C127 cell line in our laboratory was a gift from R. Axel (Columbia University, New York). C127 cells were maintained as monolayer cultures at 37°C in, essentially, Dulbecco's modification of Eagles medium. Alpha modification medium (Gibco) was supplemented with 10% Foetal Calf serum (Gibco or Sera Labs), 10u/ml penicillin and streptomycin (Gibco), 3.5mg/ml glucose and 0.5µg/ml Fe $(NO_3)_3$. Selection for G418 resistant cell lines was applied by the addition of G418 (Gibco) at a concentration of 1200µg/ml.

To ensure reproducibility of all transfection experiments, early passage cells which had been frozen in parallel were routinely used. When maintained as a non-transformed monolayer the cells were split on reaching approximately 60% confluency to guard against the

selection of spontaneously transformed cells in the population.

2.1.2.B. Splitting and replating cells

On reaching confluency in a flask, the density of cells must be reduced in order to ensure normal growth. This reduction in cell density is most easily performed by detaching the cells from the surface of the flask/ plate by treatment with trypsin.

Usually, when the medium was poured off a confluent $75cm^2$ flask (Nunc), the cells were then washed twice with 2-3mls of a trypsin solution at 37^oC and a further 3mls of trypsin was left on the cells until the monolayer had completely detached. The trypsin was then inactivated by the addition of two or three volumes of medium. A proportion of the cells were discarded or used to set up additional cultures in fresh flasks, and fresh medium (15mls for a $75cm^2$ flask) was added to these and the original flask which were then replaced in the incubator.

In the case of C127 cells, trypsinised flasks were always discarded and never reused for culturing the cell line, since C127 cells do not attach efficiently to used flasks.

Trypsin consists of: Trypsin 0.5g

Glucose 1.0g

EDTA 0.1g

Added to 50 mls of Saline D Concentrate and made up to 1 litre (pH to pH7.7 with HCl).

One litre of Saline D concentrate contains:

0.24 g Phenol Red

160.0 g NaCl

8.0 g Kcl

0.9 g $Na_2HPO_4.H_2O$

0.6 g KH_2PO_4

2.1.2.C. Mycoplasma testing

Mycoplasmas are common contaminants of all cultures. They can effect the selection for TK^+ cells after DNA transfection by utilising the thymidine present in HAT medium and converting it to thymine which cannot be utilised by mammalian cells. Essentially, Ltap207 cells contaminated with mycoplasma cannot be selected for TK^+ transformants since the medium becomes depleted of thymidine and the aminopterin component of HAT blocks de novo synthesis of deoxythymidine monophosphate.

Ltap207 cells were therefore checked for mycoplasma contamination before being set up for transformation experiments.

Mycoplasma contamination was detected by the method of Chen, T.R. (1977). Stock Ltap207 cells were trypsinised and suspended at a concentration of 1 x 10^6 cells/ml in BrdU or non-selective medium.

One or two drops of the above suspension was added to 2mls of medium in 2cm petri dishes (Nunc) with sterile coverslips placed on the bottom. The plates were agitated to spread the cells over the surface of the coverslip. They were then incubated at $37^{o}C/10\%CO_2$ for 24-28 hours. The medium was then removed from the above plates and the cells fixed to the coverslip by soaking with 2 - 3mls/fix, Methanol:Acetic Acid (3:1) and left for 2 mins. The fix was removed and the above was repeated twice, leaving the fix for 5 mins. The coverslips were then air dried and stained for 10 mins with Dulbecco's PBS containing Hoechst 33258 fluorescent stain at a concentration of .05µg/ml.

The coverslips were then washed in distilled water and mounted on slides with a drop of PBS.

The cells were visualised under the microscope and if contaminated with mycoplasma, fluorescent spots could be seen attached to the cells when viewed with a UV light source.

2.1.2.D. Microinjection of recombinant DNA molecules into mouse C127 or Ltap207 cells

The arrangement of the microscope and micromanipulator was as described by J.P. Simons (1985). C127 or Ltap207 cells were plated at a low density (1 x 10^3 cells) on 35mm petri dishes. Injections of cells were carried out as described by J. P. Simons (1985). 24 hours after plating the cells were injected in a line across the centre of the petri dish with ~1 x 10^{-11} mls of recombinant DNA in PBS at a concentration of 1-10µg/ml which was equivalent to 1-10 molecules injected per cell for a molecule of 10kb. After injection the medium on the petri dish was changed (to selective or otherwise) and the cells incubated at 37°C, 5%CO_2. The medium was changed every 3 or 4 days until foci or colonies appeared 2-6 weeks later which were then picked and expanded into two large cultures for freezing and DNA preparation. In the course of this work, all the microinjection of recombinant DNAs was performed by Dr. C. J. Bostock.

2.1.2.E. DNA mediated gene transformation of Mouse Ltap207 and C127 cells

DNA transformation experiments were performed by the calcium phoshate precipitation method of Graham and van der Eb (1973) and Graham *et al* (1980).

Briefly, a 2x DNA-$CaCl_2$ solution [1mM Tris pH8.1, 0.1mM EDTA, 250mM $CaCl_2$, 20µg of recombinant DNA per ml] was added dropwise to an equal volume of 2 x HEBS solution [2x HEBS is 50mM Hepes, 280mM NaCl, 1.5mM Na_2HPO_4, .01mM EDTA, pH7.15] while introducing air bubbles at the bottom of the tube through a Pasteur pipette. The DNA calcium phosphate precipitate was allowed to form at room temperature over a period of 30-40 minutes. Aliquots containing 30µg of recombinant DNA (1.5mls) were then added to cell cultures in $75cm^2$ flasks containing 15ml of fresh non-selective medium and these were incubated at 37°C, 10%/CO_2. The cell cultures were set up approximately 24 hours before the addition of the precipitate and were seeded with 1 x 10^6 cells per $75cm^2$ flask (Nunc). It is important to note that in all these experiments carrier DNA was omitted.

2.1.2.F. Selection of C127 focus forming cells

1 x 10^6 cells were plated on a $75cm^2$ flask in Dulbecco's modification of Eagles medium (DMEM) with 10% foetal calf serum, penicillin and streptomycin 15-24 hrs before the addition of the DNA precipitate. 24 hours after the addition of the precipitate the medium was removed, the cells were washed twice with DMEM (5mls) and 15mls of fresh DMEM were added. The medium in these

flasks was then changed every 4-5 days until the appearance of macroscopic foci, usually three to four weeks later. These were then picked and expanded into large cultures. Foci consist of morphologically transformed cells which, because of some growth advantage, are able to overgrow the flat monolayer of non-transformed C127 cells. Because of the nature of this selection system it is important that the non-transformed stock C127 cells are maintained at a low cell density to reduce the probability of spontaneous foci appearing. At no time in control transformations using plasmid pBR328 or pAT153 as the DNA source or when using a blank precipitate (no DNA included) were any spontaneous foci detected.

2.1.2.G. Selection for G418 resistant C127 transformants

Some of the recombinant plasmids used carried the aminoglycoside phosphotransferase gene of bacterial transposon Tn5 (Neo) as a genetic marker. The gene product of Neo confers resistance to the antibiotic G418, which is toxic to eukaryotic cells (Jimenez and Davis, 1980; Colbere-Garapin et al, 1981; Southern and Berg, 1982). For transformation of C127 cells to $G418^R$ (or Neo^R), the procedure followed was as for focus selection except that 48 hours after the addition of the precipitate the medium was replaced with

DMEM 10% Foetal calf serum, penicillin and streptomycin supplemented with 1.2mg/ml of G418 (Gibco). This G418 containing medium was changed every 7 days until discrete colonies appeared approximately 14 days after selection was applied. These colonies were then picked and expanded.

2.1.2.H. Selection for TK^+ Ltap207 transformants

When selecting for Ltap207 transformants, which have become TK^+ due to uptake of a recombinant molecule containing the Herpes simplex virus thymidine kinase gene (HSV-TK) (Wigler *et al*, 1977; Bacchetti and Graham, 1977; Pellicer *et al*, 1978), TK^+ colonies were selected for by their ability to grow in HAT containing medium (Littlefield, 1964). HAT medium contains aminopterin (A), which blocks *de novo* nucleotide biosynthesis, hypoxanthine (H) and thymidine (T), are two precursors that enter in the biosynthetic pathway after the blockage imposed by aminopterin. Thymidine kinase is required to convert free thymidine to dTMP so as to complete synthesis (see Figure 2.1). 48 hours later, before the addition of selective medium the cells were washed twice with non-selective medium. This non-selective medium was replaced by 15mls of HAT medium per flask. HAT medium was changed every 4-5 days until colonies appeared two weeks later and were then picked and expanded.

FIGURE 2.1. Schematic diagram of the nucleotide biosynthesis pathway.

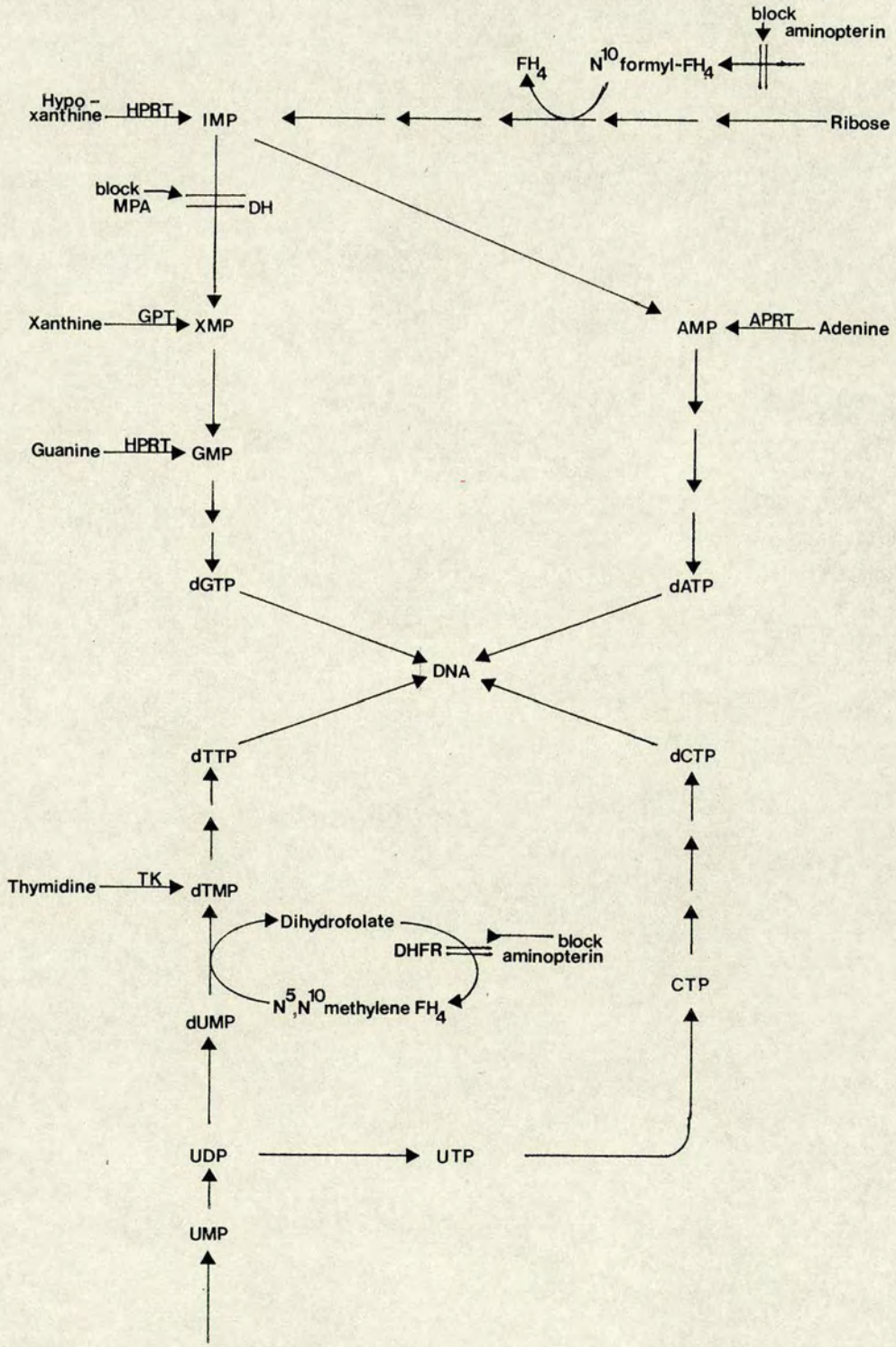
APRT : Adenosine Phosphoribosyl Transferase.

HPRT : Hypoxanthine Guanine Phosphoribosyl Transferase.

GPT : Guanine Phosphoribosyl Transferase.

TK : Thymidine Kinase.

MPA : Mycophenolic Acid.


block
aminopterin
FH4
N10formyl-FH4
Hypo-
xanthine
HPRT
IMP
Ribose
block
MPA
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Xanthine
GPT
XMP
AMP
APRT
Adenine
Guanine
HPRT
GMP
dGTP
dATP
DNA
dTTP
dCTP
Thymidine
TK
dTMP
Dihydrofolate
DHFR
block
aminopterin
N5,N10methylene FH4
dUMP
CTP
UDP
UTP
UMP

2.1.2.I. Selection for G418^R resistant Ltap207 cells

The procedure for the selection of G418^R (or Neo^R) Ltap207 cells was identical to that carried out for the selection of G418^R C127 cells, except that the medium in this case was minimal essential medium, α-modification with 10% donor calf serum, penicillin and streptomycin supplemented with 1.2mg/ml of G418 (Gibco).

2.1.2.J. Picking foci, TK$^+$ colonies and G418^R colonies

To pick foci or colonies, cloning rings were used, which consist of sterile glass tubes approximately 0.5cm in height and 0.5cm in diameter. One end of the cloning ring was dipped in sterile silicon grease and this was then placed over a focus or colony so that the centre of the focus/colony is in the centre of the ring. The cloning ring was then pressed down so that the silicon grease formed a liquid tight seal between the flask surface and the end of the cloning ring. The focus/colony was then removed from the surface of the flask by a series of washes with the trypsin solution (see previous section). These washes were placed in 5mls of the appropriate medium in a 25cm^2 flask (Nunc) which was eventually expanded to approximately 1 x 10^7 cells for purification of DNA.

In each transformation experiment at least six foci

or colonies were picked, usually three from each of two independent transformation experiments. In each case a fraction of the expanded focus/clone (cell line) was retained for freezing in liquid nitrogen. After picking foci or colonies the remaining cells on each flask were fixed and stained with giemsa R66 to determine the transformation frequency.

2.1.2.K. Transformation frequency

In most cases the transformation frequency was determined after the appearance of foci or colonies. This has been expressed in terms of the number of foci/µg of BPV-1 DNA, or number of TK^+ colonies/µg of HSV TK DNA and therefore the transformation frequencies have been corrected for differences in the sizes of the vectors. Because, for each vector tested only one precipitate (containing 60µg in 3mls) was made which was divided between two flasks of cells (1×10^6/flask), the transformation frequencies are not comparable since it is not known if these figures are reproducible. For this reason the transformation frequencies are only briefly discussed in the results section. However, tables of the various vectors introduced into mouse C127 or Ltap207 cells, or both, are presented in Tables 2.1, 2.2 and 2.3 overleaf, and the transformation frequencies obtained are indicated where determined. These Tables also indicate the bacterial host of each vector used

TABLE 2.1.

TRANSFORMATION OF C127 MOUSE CELLS BY BPV-1 CONTAINING PLASMID DNAs

Plasmid (as open circular supercoiled mixture unless stated)	Bacterial Host dam^+/dam^-	Site of Cloning of BPV-1 in plasmid	Size (kb)	Transformation Frequency* No. of Foci/μg of BPV-1 DNA
pBR328/BPV_I	dam^+	BamHI	12.85	9.4
	dam^-	BamHI	12.85	16.4
pΔBR328/BPV_I†	dam^+	BamHI	12.51	9.75
	dam^-	BamHI	12.51	11.00
BamHI cleaved†	dam^+	BamHI	7.95kb + 4.56kb	12.20
pΔIR/BPV_{II}	dam^+	BamHI	11.96	16.8
pAT153/BPV_I	dam^+	BamHI	11.6	N.D.
	dam^-	BamHI	11.6	N.D.
pΔAT153/BPV_I	dam^+	BamHI	11.26	41.2
pΔAT153/BPV_{II}	dam^+	BamHI	11.26	35.6
pΔAT153/BPV_I/EN-DUP B	dam^+	BamHI	11.32	2.75
pΔAT153/BPV_I/EN-DUP C†	dam^+	BamHI	11.32	4.63
pΔAT153/BPV_I/EN-DUP D†	dam^+	BamHI	11.32	1.0
pBR328/BPV/TK_2	dam^+	HindIII	14.95	N.D.
	dam^-	HindIII	14.95	N.D.
pBR328/TK/BPV_I	dam^+	BamHI	14.95	10.2
	dam^-	BamHI	14.95	14.3
pΔBR328/TK/BPV_I†	dam^+	BamHI	14.6	~20
	dam^-	BamHI	14.6	~20
pBRd/BPV-β_1† (D. DiMaio)	dam^+	HindIII + BamHI	16.34	95
$pCGBPV_{9\Delta B5}$† (P. Matthias)	dam^+	HindIII	11.3	4.67) 5.5)
pML2d/BPV-1†	dam^+	BamHI	10.59	34
pBR328	dam^+		4.9	0
pΔBR328	dam^+		4.56	0
pΔBR328	dam^-		4.56	0

NOTE * (1) The above transformation frequencies were obtained from duplicate plates in every case. These figures are not directly comparable since the transformation experiments were carried out with different batches of cells, media and solutions and on different days.

(2) The numbers of foci/μg of BPV-1 DNA are expressed in terms of only the BPV-1 component, and have therefore been corrected for differences in the overall size of the vectors,

$$\text{No. of foci/μg of BPV-1 DNA} = \left(\frac{\text{No. of foci}}{\text{(μg of vector DNA)}}\right) \times \left(\frac{\text{size of vector}}{\text{(size of BPV-1 DNA component)}}\right)$$

Thus although 30μg of total vector DNA was added to 1×10^6 cells in each flask, the number of cells per μg of BPV-I DNA will vary for different vectors.

(3) All transformations were performed in the absence of carrier DNA, that is the precipitate contained plasmid DNA only, at a concentration of 20μg/ml of final precipitate. 1.5mls of the precipitate were added to 15mls of medium in an 80cm² flask 15-24 hours after seeding it with 1×10^6 cells.

† - indicates that these plasmids were also microinjected into C127 cells (by C. J. Bostock) and transformants analysed.

TABLE 2.2.

TRANSFORMATION OF Ltap207 MOUSE CELLS BY HSVTK CONTAINING PLASMID DNAs

Plasmid (as open circular supercoiled mixture unless stated)	Bacterial Host dam^+/dam^-	Site of Cloning of BPV-1 in plasmid	Size (kb)	Transformation Frequency* No. colonies/μg of HSVTK DNA
$pBR328/TK_2$	dam^+		7.0	62.7
$pBR328/TK_2$ (dimeric form)	dam^-		7.0	10.3
$p\Delta BR328/TK_2$	dam^+		6.66	43.4
$p\Delta BR328/TK_2$	dam^-		6.66	31.7
$pBR328/BPV/TK_2$	dam^+	HindIII	14.95	47.6
$pBR328/BPV/TK_2$	dam^-	HindIII	14.95	57.0
$pBR328/TK/BPV_I$	dam^+	BamHI	14.95	7.6
$pBR328/TK/BPV_I$	dam^-	BamHI	14.95	22.0
$p\Delta BR328/TK/BPV_I$	dam^+	BamHI	14.6	19.0
$p\Delta BR328/TK/BPV_I$	dam^-	BamHI	14.6	13.8
pBR328	dam^+		4.9	0
	dam^-		4.9	0
$p\Delta BR328$	dam^+		4.56	0
	dam^-		4.56	0

NOTE *

(1) The above transformation frequencies were obtained from duplicate plates in every case. These figures are not directly comparable since the transformation experiments were carried out with different batches of cells, media and solutions and on different days.

(2) The numbers colonies/μg of HSVTK DNA are expressed in terms of only the HSVTK component, and have therefore been corrected for differences in the overall size of the vectors,

$$\text{No. colonies/}\mu\text{g of HSVTK DNA} = \frac{(\text{No. of colonies})}{(\mu\text{g of vector DNA})} \times \frac{(\text{size of vector})}{(\text{size of HSVTK DNA component})}$$

Thus although 30μg of total vector DNA was added to 1×10^6 cells in each flask, the number of cells per μg of HSVTK DNA will vary for different vectors.

(3) All transformations were performed in the absence of carrier DNA, that is the precipitate contained plasmid DNA only, at a concentration of 20μg/ml of final precipitate. 1.5mls of the precipitate were added to 15mls of medium in an 80cm² flask 15-24 hours after seeding it with 1×10^6 cells.

TABLE 2.3. TRANSFORMATION OF C127/Ltap207 BY BPV-1 VECTORS WHICH ALSO CARRY A G418 RESISTANCE MARKER*

Plasmid	Bacterial Host dam^+/dam^-	Site of cloning of BPV-1	Size	Neo Promoter	Host Mouse cell line C127/Ltap207	Method of Introduction Precipitation/ Injection	Selection Foci/G418
$p\Delta BR328/BPV_I/Neo_{I\rightarrow IV}$)	dam^+	BamHI	15.65	SV40 early	C127	Co-precipitate equal quantity of each	G418
$p\Delta BR328/BPV_INeo_I$	dam^+	BamHI	15.65	SV40 early	C127	Microinjection	Foci and G418
$pCGBPV_{9\Delta B5}$	dam^+	BamHI	11.3	HSV-TK	C127	Microinjection and Precipitation	Foci and G418
					Ltap207	Microinjection and Precipitation	G418
pML2d/BPV/MMTneo	dam^+	BamHI	14.6	Mouse metal-ollionein	C127	Microinjection	G418

NOTE * (1) The transformation frequency for these plasmids was not determined except in the case of focus selection for $pCGBPV_{9\Delta B5}$ (see Table 2.2).

(2) All calcium phosphate precipitations were carried out in the absence of carrier DNA.

(i.e. dam$^+$ or dam$^-$), the size of the vector and whether the vector was microinjected into the cells and the same selection applied.

2.1.2.L. Frozen storage of cell lines

Approximately 1 x 10^6 trypsinised cells suspended in medium were centrifuged at 1,000 rpm. The supernatant was removed. The cell pellets were resuspended in 0.5mls of freezing medium which was then transferred to a freezing vial (Nunc). These cell suspensions were placed in a polystyrene container at -70^{o}C and allowed to freeze overnight. The freezing vials were then transferred to a liquid nitrogen storage tank.

Freezing medium contains: 95% Foetal Calf Serum
5% Dimethyl Sulphoxide

2.2. Preparation of DNA from Procaryotic and Eucaryotic sources

2.2.1.A. Preparation of plasmid DNA from bacteria - large scale alkaline lysis method

Plasmid DNA was prepared from E.coli cultures as described by Birnboim and Doly (1979).

500mls of innoculated L-broth, containing the appropriate antibiotic, were incubated at 37°C at 200 rpm for approximately 18 hours. The culture was then transferred to 2 x 250ml polysulfone bottles (Sorvall) and the cells pelleted at 5k rpm at 4°C for 5 min.

The cell pellets were resuspended in 9mls of 50mM Glucose, 25mM Tris HCl (pH8.0), 10mM EDTA, and the contents pooled. Lysozyme (Sigma) was then added in 2mls of the Glucose solution at a concentration of 40mg/ml and the lysate left at room temperature. After 15 minutes 40 mls of 0.2MNaOH, 1% SDS were added. The mixture was swirled and placed on ice for five minutes, after which 20mls of cold 5M KOAC pH4.8 were mixed in and reincubated on ice for 15 minutes.

The resulting precipitate was spun down at 8k rpm for 10 minutes and the supernatant was filtered through muslin gauze into a graduated cylinder. This supernatant was then transferred to a 250ml stainless steel bottle (Sorvall) and 0.6 volumes of iso-propanol were mixed in. The precipitate was collected by centrifugation at 8k rpm for 5 minutes. The supernatant was poured off and the pellet drained by placing the bottles upside down for 30 minutes. When thoroughly dry the pellet was resuspended in 10mls of 10mM Tris HCl pH7.5 10mM EDTA and neutralized to a pH of 7.5 by the addition of approximately 200µl of 2M Tris base.

The volume was then made up to 12 mls by addition of 10mM Tris (pH.7.8) 10mM EDTA, 13.4g of caesium chloride were added and when dissolved, 1.4mls of an ethidium bromide solution (10mg/ml) mixed in to give a final density of 1.55 g/ml (refractive index = 1.386). This solution (17 mls total volume) was then loaded into a 17ml polyallomer tube (Sorvall) which was placed in a Sorvall OTD865B rotor and centrifuged at 40k rpm at 20°C for 24-48 hours in an OTD50 ultracentrifuge (Sorvall). When the run was complete the tube was placed under a longwave U.V. lamp (300n m) and the plasmid band visualized. The plasmid was removed by using a syringe to pierce the tube and pull out the appropriate band.

To remove the ethidium bromide from the DNA, an equal volume of iso-propanol saturated with caesium chloride was added. The mixture was shaken and the upper phase removed after centrifugation at 2,000 rpm for one minute. This was repeated 3-4 times until the lower phase became colourless. The plasmid DNA was then precipitated, by the addition of three volumes of 70% ethanol, and spun down 4k rpm, 15 mins. 4°C. The pellet was washed in 70% ethanol and resuspended in 300µl of 10mM Tris-HCl (pH7.8) 1mM EDTA (TE) and transferred to an eppendorf tube. The DNA was again precipitated by the addition of one-tenth volume of 3M NaOAC pH5.2 and 2 volumes of absolute alcohol. The pellet was again washed with 70% ethanol and finally resuspended in an appropriate volume of TE to give a

final concentration of 1-2mg/ml.

2.2.1.B. Preparation of plasmid DNA by the mini-scale rapid boiling method

The method used was essentially that described by Holmes and Quigley (1981), it was used for checking the identity of new constructs before performing a large scale plasmid preparation. 5mls of culture medium containing the appropriate antibiotic were innoculated with a single bacterial colony. This was incubated at 37^{o}C at 200rpm overnight. 1.5ml of the culture were transferred to an eppendorf tube and the cells pelleted by centrifugation for 30 seconds in an microfuge. The medium was removed and the pellet resuspended in 350µl of STET (STET = 8% sucrose, 0.5% Triton X-100, 50mM EDTA pH8.0 and 10mM Tris-HCl pH8.0). 25µl of a freshly prepared solution of lysozyme were added (10mg/ml in STET). The tube was then placed in a boiling water bath for 40 seconds after which it was centrifuged for 20-30 minutes in a microfuge. The gelatinous pellet was removed with a toothpick. The DNA was precipitated from the supernatant by the addition of one volume of isopropanol, mixed, and placed in liquid nitrogen for 5 minutes. The precipitate was sedimented by centrifugation for 10 minutes in a microfuge. The resulting pellet was washed with ether and then with 70% ethanol. The final pellet was resuspended in 50µl of TE

containing RNase (50µg/ml).

2.2.2. Isolation of total cellular DNA from mammalian cell lines

Total cell DNA was isolated by the method of Wigler et al (1979b) with some modification.

Approximately 1 x 10^7 cells were trypsinised and washed out of 75cm^2 flask with medium into a sterile 15ml Sarstedt polypropylene tube. Cells were pelleted at 1000xg for 10 min. The pellet was resuspended in 10mls of PBS and again the cells were pelleted at 1000 xg. This pellet was then resuspended in 20-40 volumes of 10mM Tris Hcl pH8.2, 100mM NaCl, 10mM EDTA, 0.5% SDS and pronase (CaLbiochem-Behring Corpn) was added to 100µg/ml. The lysate was incubated at 37°C for 12-16 hours and then extracted twice with one volume of Phenol (pH>7.6 + .1% w/v 8- hydroxyquinoline as prepared in Maniatis et al (1982)) and 0.5 volumes of 24:1 chloroform:iso amyl alcohol followed by one extraction with an equal volume of chloroform:iso amyl alcohol. The total DNA + RNA was isolated by adding one- tenth volume of 3M NaoAc pH5.2 and 2 volumes of ethanol and incubating at -20°C for one hour. The resulting precipitate was pelleted and washed with 5mls of 70% ethanol. The DNA was then dissolved in 10mM Tris pH7.5 1mM EDTA (TE).

For digestion with restriction endonucleases the DNA was dialysed against TE overnight at 4°C. The DNA + RNA concentration was estimated by OD_{260}. It was then assumed that the DNA represented two-thirds of the OD_{260}.

2.3. Analysis of DNA

2.3.1. Restriction endonuclease digestion of DNA

Various commercially available restriction endonucleases were required in the course of this work. They were BamHI, BclI, BglII, DpnI, EcoRI, HindIII, HpaI, KpnI, PvuII, Sau3A and XbaI and were purchased from either Amersham International plc, or Bethesda Research Laboratories (BRL) or Boehringer Mannheim GmbH, or New England Biolabs.

Restriction endonuclease digests were carried out in sterile eppendorf tubes using the high salt, medium salt or low salt core reaction buffers (Maniatis et al (1982)) in accordance to the manufacturers' recommendations.

High salt buffer: 100mM NaCl, 50mM Tris (pH7.5), 10mM $MgCl_2$, 1mM dithiothreitol

Medium salt buffer: 50mM NaCl, 10mM Tris (pH7.5), 10mM $MgCl_2$, 1mM dithiothreitol

Low salt buffer: 10mM Tris (pH7.5), 10mM $MgCl_2$, 1mM dithiothreitol

Digests were performed at 37°C with the exception of BclI digests which was incubated at 60°C. The concentration of DNA in digests varied from 10µg/ml to 0.5mg/ml.

The amount of enzyme used was usually the equivalent to the number of units required to digest that amount of DNA completely in one hour. Most reactions were carried out under conditions which theoretically give at least three fold excess digestion.

When digesting total genomic DNA from mammalian cell lines usually between 0.2 and 0.5 units of enzyme were used to digest 1µg of DNA and the reaction mix was incubated at 37°C for at least 12 hours to ensure complete digestion. Restriction endonuclease reactions were normally terminated by heat inactivation of the enzyme at 70°C for 10 min. Some enzymes are refractory to heat inactivation and in these cases the reaction was terminated by the addition of EDTA to a final concentration of 10mM.

When digestion of the same DNA sample was required with two different enzymes, the sample was incubated with both enzymes in the same reaction mix, if their buffers were compatible. If the two enzymes had incompatible

incubation conditions, digestion was carried out with the enzyme requiring the lower salt buffer first and then with the second enzyme after the addition of the appropriate amount of salt.

2.3.2. Shearing total genomic DNA

The analyses of cell lines transformed with bovine papilloma virus DNA containing vectors must involve a method for identifying episomal forms. The standard method for resolving extrachromosomal replicating episomes in mammalian cells is to analyse undigested DNA by electrophoresis. However, the size of DNA fragments isolated in the total cell DNA preparation can range up to 200kb in size. Since all DNA molecules above approximately 40-50kb are excluded from resolving on agarose gels and therefore run together, it is a possibility that the migration of smaller extra-chromosomal molecules would be retarded by becoming trapped in a network of high molecular weight DNA molecules. For this reason, when analysing genomic DNA for the presence of episomal forms the DNA solution was passed through a 25g syringe 6 to 8 times. This breaks up the large chromosomal molecules sufficiently to allow the unretarded passage of the smaller episomal molecules.

Usually 5µg of total DNA was made up to 30µl in dH_2O and 1 x loading buffer in a sterile Eppendorf tube.

This mix was then passed through a sterile 25 gauge syringe needle (Becton-Dickinson Microlance) using a 1ml sterile syringe (Becton-Dickinson Plastipak). This sheared DNA was then loaded directly onto an agarose gel.

2.4. Gel Electrophoresis

2.4.1. Agarose Gels

Verticle gel apparatuses of various agarose concentrations (Miles) (0.4-2%) and dimensions were used to separate DNA fragments of differing size from each other as described by Sealey and Southern (1982). In most cases the gels were run in 1 x E buffer (1 x E = 26mM Na_2HPO_4, 3.3mM NaH_2PO_4, 1mM EDTA pH 7.5- 8.00), however when one wished to isolate specific DNA fragments (see later section) the gels were run in 1x TAE buffer [1x TAE = 40mM Tris-acetate, 1mM EDTA pH8.0] and occasionally in such cases low melting point agarose was used (Sea plaque, FMC Corporation).

All gels were 3mm thick. Gels of dimensions 8cm x 8cm were used for checking digests, ligations, mini plasmid preparations etc. Gels of dimensions 12cm length x 18cm width, 18cm length x 8cm width and 18cm length x 18cm length were used for more careful analyses involving transfer to nitrocellulose and hybridisation or isolation

of specific DNA fragments.

In general, slots were approximately 4mm in width onto which variable amounts of DNA were loaded. Up to 5µg of total genomic DNA could be loaded on a 4mm slot. DNA samples at concentrations of less than 1µg/µl were loaded directly onto gels by adding one tenth volume of the follow 'stopmix' (or loading buffer): 30% Ficoll (Pharmacia Fine Chemicals), 0.25% orange G, 0.5M EDTA and 10x electrophoresis buffer. The Ficoll increases the sample density and the orange G makes the sample easily visible and acts as a marker close to the migrating front of lower-size DNA fragments.Two dimensional gels were used to distinguish super-coiled from open circular and linear episomal forms. In this case a track of 15cms length from the first dimension was cut from a 8 x 18cm gel turned through 90^{o} and molded horizontally into a 16cm (width) slot on an 18 x 18cm gel and so run in a second dimension.

After electrophoresis gels were removed from the apparatus and placed in a staining tray containing the running buffer with ethidium bromide (Sigma) at a concentration of 0.5µg/ml, the DNA in the gel was stained within 15 mins. Gels were then transferred to a black perspex plate and illuminated from above with unfiltered shortwave ultraviolet (254nm) lamps (Mineralight). The fluorescent DNA was then photographed using a Polaroid MP4 land camera on Ilford FP4 or Kodak Technical Pan film

through a red filter (Hoya 25A). The film was developed in Ilford Microphen developer with nitrogen agitation, the developing process was stopped in 5% acetic acid and fixed in Amfix (May and Baker).

Gels containing labelled DNA were dried down onto DE81 paper (Whatman) under suction in a vacuum press. On most agarose gels a HindIII digest of λ DNA was used as a molecular weight marker and as a check for aberrant running of the gel.

2.4.2. Polyacrylamide gels

Polyacrylamide gels were used for two purposes firstly, checking linker-kinasing reactions (see later) and secondly isolating small DNA molecules (<1kb) from gels. Occasionally, they were used for checking the sizes of small (<1000bp) fragments in plasmid DNA digests.

Polyacrylamide gels were made from the following stock solutions, 30% polyacrylamide in dH_2O [29:1, polyacrylamide:N'N' Methylene bis-acrylamide (BDH electrophoresis grade)], 10% ammonium persulphate (fresh solution), TEMED (Eastman-Kodak) and 10x TBE (1x TBE = 89mM Tris-borate, 89mM Boric Acid, 2mM EDTA pH8.3).

Polyacrylamide gels consisted of variable percentage acrylamide, 1x TBE and 0.067% ammonium persulphate which

was degassed and then ~50µl of TEMED were added per 100mls of degassed mix. The gel was poured immediately into a vertical apparatus with a 1.5% Agarose, in 1x TBE, plug. Gels normally set within 15 min. at room temperature. Electrophoresis was performed in 1x TBE (pH8.3).

Gels were 1mm (for checking linker kinasing) or 3mm (isolating small DNA fragments) thick with slots between 8 and 50mm depending on the amount of DNA loaded. The sample was loaded in 1x TBE that contained 2% Ficoll and 0.5% bromophenol blue and Xylene cyanol blue as molecular weight markers. The rate of migration of these dyes depends on the percentage of the polyacrylamide gel.

Polyacrylamide gels were stained and photographed as described for agarose gels. For autoradiography the gel was left stuck to the front glass plate of the gel apparatus, covered with cling film and placed in an appropriate sized cassette.

2.4.3. Isolation of DNA fragments from Agarose gels

For isolating specific sized restriction fragments for cloning into vectors or using as radioactive probes agarose gels were made up, and run in 1x TAE. Gels were stained briefly (5 mins) with ethidium bromide and the fluorescent bands visualised by placing the gels onto a

long wave ultraviolet (300nm) transilluminator on a UV transparent perspex sheet (Plexiglass 218). The band(s) of interest was excised from the gel and placed in a dialysis bag with 3-4mls of 0.5 x TAE. The DNA in the gel slice was then electroeluted (Yung et al, 1979) into the 0.5x TAE by electrophoresis on a horizontal gel apparatus in 1x TAE at 100V for two hours, to ensure all the DNA had left the gel slice. The polarity of the electrodes was then reversed for one minute to unstick the eluted DNA from the dialysis membrane. The 0.5x TAE was then transferred from the bag to a 13ml polypropylene tube (Sarstedt). The dialysis bag and gel slice were washed twice with 2-3mls. of TE to remove any remaining DNA. These washes were placed into the same polypropylene tube as above. This electroelutant plus TE was then made up to 0.2M NaCl by the addition of an appropriate amount of 5MNaCl and then passed through a primed Elutip-d mini-column (Schleicher and Schuell) which binds up to 100µg of DNA of 50bp to 50,000bp. The column was then rinsed with low salt buffer (0.2M NaCl, 20mM Tris NCl pH7.5, 1mM EDTA). The bound DNA was then eluted from the Elutip-d mini-column, by passing through 400µl of High salt buffer (1.0M NaCl, 20mM Tris-HCl pH7.5, 1mM EDTA) into a 1.5ml Eppendorf tube. The DNA recovered was then precipitated by the addition of two volumes of ethanol and incubation at -70°C, spun down in a microfuge and the DNA pellet resuspended in an appropriate volume of TE buffer.

Occasionally, low melting point 1x TAE agarose gels were used especially for isolating small quantities of DNA (50-100ng). Slices from these gels were treated similarly except that the gel slice was dissolved in a quantity of low salt buffer (roughly 5x the volume of the gel slice) and heated at 68°C for 1 hour to melt the agarose gel, cooled to 37°C and then passed through an Elutip-d column to bind the DNA at 37°C. The rest of the procedure was identical to the electroelution method except that all buffers were used at 37°C.

2.4.4. Isolation of DNA fragments from polyacrylamide gels

For isolation of bands between 0.05 and 2kb a 3.5% polyacrylamide gel run in 1x TBE was used. The dye (Bromophenol blue) was run approximately 30 centimetres. The polyacrylamide gel was stained briefly with ethidium bromide and the bands then visualised in the same manner as when isolating DNA from agarose gels. The required band(s) was excised from the gel and crushed into a smooth paste in a 13ml polypropylene tube (Sarstedt) using a siliconized glass rod. Approximately three volumes of 10mM Tris (pH7.5), 1mM EDTA were added to the paste and the DNA was allowed to elute overnight at 37°C. The polyacrylamide was spun out of this mix and the supernatant containing the DNA removed. The

polyacrylamide pellet was washed with three volumes of 10mM Tris (pH7.5) 1mM EDTA and again spun out, the supernatant from this spin was added to the previous one. This supernatant was then reduced in volume to approximately 400µl by extraction with 2-butanol, and then precipitated in an eppendorf tube with two volumes of alcohol. The DNA pellet was resuspended in 10mM Tris pH7.5 1mM EDTA and further purified by passage through an Elutip-d mini-column (Schleicher and Schuell) as previously described.

2.5. Labelling of DNA

2.5.1. Nick Translation

Radioactive probes for hybridisation to DNA immobilised on nitrocellulose filters were prepared essentially as described by Rigby et al (1977).

E.coli DNA polymerase I repairs nicks in double stranded DNA by adding a nucleotide residue to the 3' hydroxyl terminus of such nicks. In addition it also removes nucleotides from the 5' end of a nick as a consequence of exonuclease activity. The net result of these combined activities is elimination of nucleotides from the 5' end and sequential addition to the 3' end and therefore movement of the nick along the DNA (Kelly et al, 1970).

Typical reaction mixes contained 0.1µg of DNA in a total volume of 50µl. This 50µl mix contained 5µl of 10x nick translation buffer (10x NT = 500M Tris HCl (pH7.8), 50mM $MgSO_4$, 0.1M β-mercaptoethanol), 1µl each of 0.1mM dATP and dGTP (Sigma), 10µCi each of [α-^{32}P] dCTP and dTTP (410Ci/mMole, 10 mCi/ml. Amersham International plc), 0.1µg of DNA, 1µl of DNaseI (0.1µg/ml - freshly diluted from 1mg/ml stock). Sterile distilled H_2O (to make volume to 50µl) and 1-3 units of E.coli DNA polymerase I (Amersham International plc). The nick translation reaction was incubated in a 16°C water bath for up to two hours.

The percentage of the labelled nucleotides added to the mix which were incoprorated into the DNA during the course of the reaction were estimated using Tricholoro acetic acid (TCA) precipitation (Furlong, 1967). Briefly, 0.5µl aliquots from the reactions were spotted onto Whatman GF/A microfibre filters and the number of counts per minute (cpm) emitted monitored at a standard distance with a Geiger counter. The filter was then washed through with 10% TCA and again the cpm monitored from the same distance. The percentage of counts remaining on the filter gave a measure of the percentage incorporation. Normally ^{32}P-labelled probes were prepared with a specific activity greater than 10^8dpm/µg.

The reaction was terminated by the addition of 50µl of TE. The nick translated probe was then extracted once with phenol/chloroform. 10µg of yeast tRNA were added as carrier, and precipitated with the addition of 2.5M NH_4OAC to 2M and two volumes of ethanol (mix placed in liquid N_2 for two minutes). After centrifugation, the pellet containing the ^{32}P labelled DNA was resuspended in 100µl of TE and stored for up to 24 hours at 4^oC for use in hybridisations.

2.5.2. Labelling restriction fragments by filling recessed 3' ends with the Klenow fragment of E.coli DNA polymerase I

The reaction conditions were identical to those used for nick translation reactions (see last section) except only the Klenow fragment of E.coli DNA polymeraseI was used, no DNAase was used, usually only one type of labelled dNTP was used and the dNTP's added to the reaction mix depended on the sequence of the 5' overhang at the site of restriction.

The 3' recessed ends of restriction fragments were filled in using DNA polymeraseI Klenow fragment (Amersham International plc). A 50µl reaction mix contained 5µl of 10x nick translation buffer, 1µl of the required [$\alpha^{32}P$] labelled dNTP (410Ci/mM, 10mCi/ml, Amersham International plc) or 0.1mM unlabelled dNTP's

(Sigma), 0.5-5µg of the DNA fragment and 1-3 units of Klenow fragment. Distilled H_2O was added to make the final volume 50µl and the reaction was incubated at 25°C for 30 minutes and stopped by placing on ice. Incorporation was monitored by TCA precipitation. The labelled DNA was precipitated from the mix by the addition of four volumes of 2.5M NH_4AC and two volumes of ethanol. After centrifugation the pellet was resuspended and stored in TE at -20°C.

2.6. Transfer of DNA from agarose gels to nitrocellulose filters

Transfer of DNA to nitrocellulose was essentially as described by Southern (1975). After electrophoresis was completed, the DNA in the gel was stained with ethidium bromide and photographed under ultraviolet light, as previously described (section 2.4.1). In many cases during the course of this work sheared or undigested DNA was run on agarose gels. As a result the tracks contained a large amount of material of a high molecular weight (>30kb). To ensure transfer of this material to nitrocellulose, gels were normally exposed to U.V. light for a total of seven minutes. The extended U.V. exposure introduces sufficient single stranded nicks into the high molecular weight DNA so that when denatured it is transferred to nitrocellulose efficiently.

After exposure to UV the DNA was denatured by soaking the gel in several volumes of 1.5M NaCl and 0.5M NaOH for 45 minutes at room temperature. The gel was then neutralized by soaking in several volumes of 1M Tris-HCl (pH4.5) and 1.5M NaCl for 1.0 hours, at room temperature.

The gel was then placed onto the surface of a blotting apparatus freshly soaked with 20 x SSC. The edges of the gel were prevented from touching the blotting surface by carefully placing strips of Nescofilm under the gel which could be manoeuvered into position. A nitrocellulose filter (Schleicher and Schuell) was then floated on 0.1% SDS until the surface was completely wet, immersed for a few minutes, rinsed several times in distilled H_2O then soaked in 2 x SSC. This nitrocellulose filter was placed carefully on top of the gel so that no air bubbles were formed. A piece of Whatman 3mm paper (soaked in 2 x SSC) was laid over the nitrocellulose, a second dry piece of Whatman 3mm was placed on top and paper towels (Kim Dri) were then stacked on the 3mm paper and weighed down with a glass plate. Transfer of DNA was allowed to proceed for 12-24 hours after which the filter was removed from the blotting apparatus, rinsed in 4 x SSC, placed between two sheets of dry 3mm paper and baked for two hours at $80^{o}C$ under vacuum.

2.7. Hybridisation of radioactive probes to DNA immobilised on Nitrocellulose

The baked filter from above was floated on the surface of 4 x SSC until its upper surface was wet. It was then immersed in the 4 x SSC for a few minutes and transferred to a hybridisation tube. The filter was then incubated at 68°C with 25mls of prehybridisation solution (see below) for 3-6 hours on a rotating rack, after which the prehybridisation solution was replaced by 10mls of hybridisation solution (preheated to 68°C) (see below) containing heat denatured ^{32}P-labelled probe. The hybridisation tube was reincubated at 68°C for 15- 24 hours, after which the filter was floated out of the tube with cold 4 x SSC. Unhybridised probe was removed by sequential washes in 3 x SSC (once), 1x SSC (twice) and 0.1x SSC (twice) with 0.1% NaPPi and 0.1% SDS at 58°C for 30 minutes.

The filters were then air dried for 30 minutes and put down for autoradiography.

Prehybridisation solution

5 x SET

5 X DENHARDTS

0.1% SDS

0.1% NaPPi

Heat denatured Salmon Sperm or Calf Thymus DNA (1mg/10mls).

Hybridisation Solution

As above, but with 10% Dextran Sulphate and heat denatured α^{32}P-labelled DNA probe (0.1µg/10mls).

20x SET: is 3M NaCl, 0.4M Tris-HCl (pH7.8), 20mM EDTA.

50x Denhardts solution is:

1% Ficoll, 1% polyvinylpyrrolidone

1% Bovine Serum Albumin

2.8. Autoradiography

Radioactive ^{32}P-labelled nucleic acids hybridised to nitrocellulose or run on gels were detected by autoradiography. The material to be autoradiographed was stuck onto cardboard or Whatman 3mm paper which was then wrapped in Cling Film, stuck down in an X-ray film cassette and covered with a preflashed sheet of X-ray Film (Fuji RXNIF). The film was then covered with an Ilford fast tungstate intensifying screen and stored at -70^{o}C for the required exposure time. The combination of preflash, intensifying screens and exposure at -70^{o}C can increase the sensitivity of detection of ^{32}P eight-fold to ten-fold (Laskey and Mills, 1977). Autoradiographs were developed in an Agfa-Gevaert automatic X-ray film processor using Agfa-Gevaert G153 developer and E353 fixative.

2.9. Purfication of circular DNA forms from transformed cell lines on caesium chloride; ethidium bromide gradients

In some cases, selected cell lines were chosen for isolation of circular DNA forms to further characterise the structure of exogenously added DNA sequences in transformed cell lines. On the first attempt, 50µg of total cellular DNA (prepared in the normal way see Section 2.2.2) were diluted in 3.5ml of TE and shaken for two hours at room temperature. 4g of caesium chloride were added, and when dissolved 0.5mls of an ethidium bromide solution (10mg/ml) were added giving a final solution of density approximately 1.55 g/ml and refractive index of 1.386 (as in normal plasmid preparations). The gradients were placed in 4.2ml polyallomer tubes (Sorvall) and loaded onto a Sorval OTD850 rotor and spun at 40k rpm for 48 hours. The remaining 0.8mls of DNA/CsCl/EtBr was retained as a control for nicking of supercoiled molecules during subsequent processing, this aliquot was therefore labelled PRE (Pregradient).

Gradients were removed from the rotor and fractionated from bottom to top. Approximately 15 x 300µl fractions were collected in eppendorf tubes, the ethidium bromide removed by 3x extraction with isopropanol/caesium chloride, the DNA precipitated by addition of three volumes of 70% ethanol and 50µg of tRNA as carrier. The

resulting pellet was washed with 70% ethanol and resuspended in 500µl of TE overnight at 4°C. The DNA was then again precipitated with 1/10th volume 3M NaOAC (pH5.8) and two volumes of absolute alcohol, pelleted, washed with 70% ethanol, and resuspended 100µl of TE. The PRE aliquot(s) were treated in a similar manner. (All this processing was necessary to eliminate salt effects when fractions were run on agarose gels.) A sample of each fraction was run on a 0.6% agarose gel with the 'PRE' sample, total cellular DNA, and the appropriate undigested plasmid as markers.

As a variation, in one case a total cell lysate (using Sarkosyl in place of SDS) from a semiconfluent $25cm^2$ flask, was pronased for four hours and then loaded onto a similar gradient. The purpose of this variation was to distinguish between material in the open circular fraction generated through nicking and breakage of supercoils, and normally open circular and/or linear forms.

2.10. ATP-dependent Deoxyribonuclease (ExoV)

This enzyme, ExoV, was a gift from M. Anai (Kyushu University, Fukuoka, Japan) and was made by Toyobo Co. Ltd. ExoV is prepared from *Micrococcus luteus* and its activity is such that in the presence of ATP it digests linear double stranded and single stranded

DNA to yield acid soluble nucleotides (Anai et al, 1970). Recently Yamagishi et al (1983) used this enzyme to prepare polydisperse circular DNA from mouse thymocytes. The enzyme does not digest open circular or supercoiled plasmid forms.

In this work the enzyme was used to distinguish linear and circular forms of Bovine papilloma virus DNA present in transformed cell lines.

The DNA (0.1-5µg) was digested in a total volume of 50µl containing 1x ExoV buffer (10x ExoV buffer = 0.667M Glycine-NaOH pH9.4, 0.3M $MgCl_2$, 83mM β-mercapto-ethanol), 1mM rATP (fresh) and the required amount of ATP-dependent DNase (1 unit produces 10nmoles of acid soluble nucleotides from 20nmoles of E.coli ^3H DNA in 30 minutes at 37°C). For running on agarose gels, the DNA was precipitated in 0.3M NaOAc (pH5.8) and 2 volumes of ethanol, pelleted, washed with 70% ethanol and resuspended in 20µl of TE.

2.11. Analysis of Chromosomes prepared from cell lines

2.11.1. Chromosome Preparation

Chromosomes were prepared by using a slight modification of a described method (Young et al, 1981). Cultures of C127 BPV-1 transformed cells from ten (60-75%

confluent) 175cm^2 flasks were exposed to colchicine at a final concentration of 2 x 10^{-6}M for not more than 16 hours. The mitotically arrested cells were shaken off the monolayer and suspended in the culture medium. These arrested cells were harvested by centrifugation at 400g for 5 mins. The cell pellet was resuspended in 50mls of 0.075M KCl and incubated for 15 min. at 37^{o}C, the cells were again harvested by centrifugation at 400g for 5 mins. The cell pellet was then resuspended in 50mls polyamine buffer (15mM Tris/ 2mM EDTA/0.5mM ethylene glycol bis (β-aminoethyl ether)-N, N, N',N'-tetra acetic acid/80mM KCl/20mM NaCl/14mM 2-mercaptoethanol/0.2mM Spermine/0.5mM Spermidine, pH7.2), the cells were again centrifuged at 400g for 5 mins and the pellet resuspended in 10mls of the polyamine buffer containing 0.1% digitonin (made up as fresh and filter sterilised stock). The suspension was then vortexed at high speed for approximately 1 minute to lyse the mitotic cells and was then placed on ice and forced through a 25 gauge syringe needle twice.

2.11.2. Fractionation of chromosomes by zonal Centrifugation

The procedure used for fractionation of chromosomes from the above mitotic cell lysate was essentially as described by Padgett et al (1977) and Tyler-Smith and Bostock (1981) but with slight modifications. The above

suspension was made up to 40mls with a final sucrose concentration of 5% (w/v) in the polyamine buffer. This suspension was then loaded at 600rpm onto a preformed 20% to 45% sucrose gradient with a 50% sucrose cushion in a low speed MSE zonal rotor at 4°C. All sucrose solutions were made up in the polyamine buffer and were at 4°C. When loading was complete the sample was overlayed with 2% sucrose solution in polyamine buffer, the rotor was accelerated to 3500rpm and held at that speed for 40min. The rotor was then decelerated over five minutes to 600 rpm and the gradient fractionated from top to bottom by pumping 50% sucrose in from the bottom at a rate of 100mls/min. The gradient was fractionated into 38 fractions of 40mls. Any small extra-chromosomal material was retrieved from the six uppermost fractions by centrifugation of 14ml aliquots on the MSE Ti50 (14ml buckets) rotor at 38,000rpm overnight. To retrieve large 'chromosomal' material from the remaining fractions, these were centrifuged at 14,000 rpm in an SS-34 rotor in a Sorvall RCB2 centrifuge. The supernatants were poured off and stored in separate tubes, and the pellets drained and resuspended in 100µl of the polyamine buffer by vortexing vigorously. Small portions from fractions Nos. 9, 21, 27 and 33 were removed for visualisation of chromosomes, however, in no case could chromosomes be detected after fixing and staining. This may have been due to loss of a large quantity of material into the 50% cushion due to clumping of chromosomes in the large scale preparation. The

remainder of each fraction was used for the extraction of DNA by pronasing, phenol:chloroform extraction (1x), chloroform extraction (1x) and precipitation with 0.3M NaoAC pH7.2 and 2x vol of ethanol. The resulting DNA precipitate was microfuged, the pellet washed with 70% ethanol and resuspended in 50µl of 10mM Tris 1mM EDTA (pH7.5). 25µl aliquots of undigested DNA from every even numbered fraction was then anlysed by electrophoresis on a 0.65% agarose gel which was then transferred to nitrocellulose (Section 2.6).

It must be pointed out that due to the scale of the above fractionation procedure the workload was shared equally between Dr. C. J. Bostock and myself.

2.12. Recovery of plasmids containing BPV-1 from mammalian cell DNA by bacterial transformation

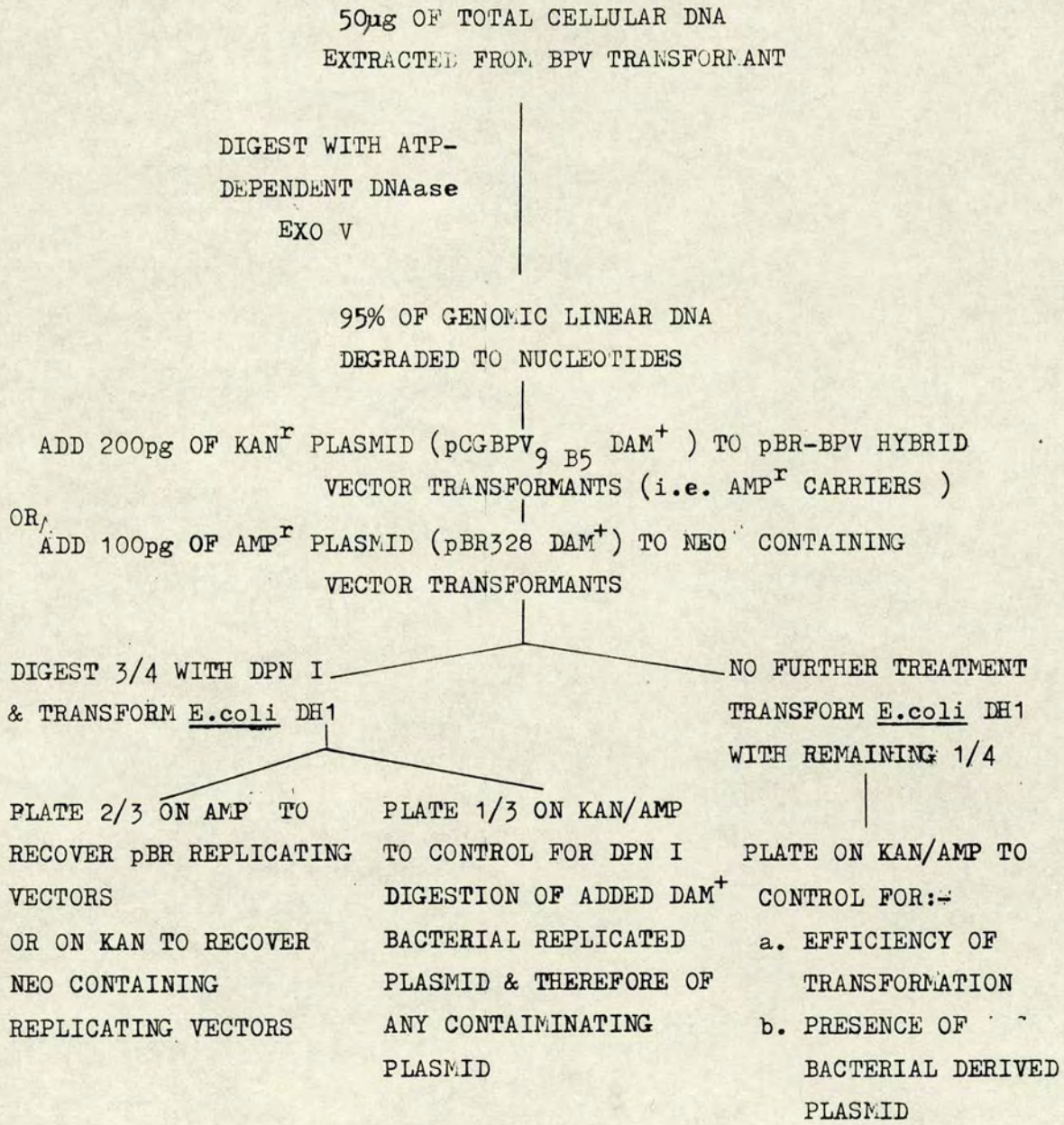
In many cases in the course of the work, transformed cell lines were generated which, upon blot hybridisation analyses, were seen to contain extrachromsomally replicating episomes indistinguishable from the input vector DNA. In order to investigate these vectors further an attempt was made to recover these plasmids into E.coli DH1 by using a transformation protocol that can yield up to 1×8^8 transformants per µg of pBR322 (Douglas Hanahan, 1983), the competent DH1 cells

were prepared in collaboration with R.Hill, MRC CAPCU. The recovery procedure is summarised in Fig. 2.2 and described below.

Because previous attempts at such experiments had failed and suggested that high molecular weight mammalian chromosomal DNA in large excess reduced the transformation frequency, digestion of total cellular DNA (50µg) with the ATP-dependent DN'ase ExoV (see Section 2.10 above) was used to remove such high molecular weight DNA and enrich for the extrachromosomal open and supercoiled circular vector forms.

After ExoV digestion the remaining DNA was precipitated by the addition of 5M NH_4Ac to a final concentration of 2M and 2x volumes of ethanol. The pellet was resuspended in 15µl of TE to which 200pg ($pCGBPV_{9\Delta B5}$-Kan^R) or 100pg (pBR328 $Amp^r/Tet^R/Cam^R$) of plasmids derived from <u>dam</u>$^+$ bacteria were added depending on the antibiotic resistance expected to be carried by the putative recoverable replicating vector in the mammalian DNA (i.e. $pCGBPV_{9\Delta B5}$ kan^R was added to DNA where the recoverable vector carried an Amp^R marker, pBR328 to those which carried the kan^R gene). To control for the presence of contaminating non-mammalian cell replicated vector in these DNA preparation, 3/4 of each preparation (i.e. 37.5µg equivalent of starting material) was digested with DpnI, and used to transform <u>E.coli</u> DH1, 2/3 of the

FIGURE 2.2. Flow chart for strategy used to recover replicating vector from total mammalian cellular DNA into competent E.coli.


50µg OF TOTAL CELLULAR DNA
EXTRACTED FROM BPV TRANSFORMANT
DIGEST WITH ATP-DEPENDENT DNAase EXO V
95% OF GENOMIC LINEAR DNA DEGRADED TO NUCLEOTIDES
ADD 200pg OF KAN^r PLASMID (pCGBPV$_{9\ B5}$ DAM$^+$) TO pBR-BPV HYBRID VECTOR TRANSFORMANTS (i.e. AMP^r CARRIERS)
OR
ADD 100pg OF AMP^r PLASMID (pBR328 DAM$^+$) TO NEO CONTAINING VECTOR TRANSFORMANTS
DIGEST 3/4 WITH DPN I & TRANSFORM E.coli DH1
NO FURTHER TREATMENT TRANSFORM E.coli DH1 WITH REMAINING 1/4
PLATE 2/3 ON AMP TO RECOVER pBR REPLICATING VECTORS OR ON KAN TO RECOVER NEO CONTAINING REPLICATING VECTORS
PLATE 1/3 ON KAN/AMP TO CONTROL FOR DPN I DIGESTION OF ADDED DAM$^+$ BACTERIAL REPLICATED PLASMID & THEREFORE OF ANY CONTAIMINATING PLASMID
PLATE ON KAN/AMP TO CONTROL FOR:-
a. EFFICIENCY OF TRANSFORMATION
b. PRESENCE OF BACTERIAL DERIVED PLASMID

transformation mix (25μg equivalent) was placed on selection for the putative recoverable vector, the remainder was plated so as to select for any remaining intact added *dam*$^+$ plasmid. The remaining material, untreated with DpnI (12.5μg equivalent) was used to separately transform DH1 and to select for the added *dam*$^+$ plasmid and thus measure the transformation frequency in each individual transformation.

Testing the sensitivity of the transforming DNA to DpnI rules out the possibility that the BPV plasmids which were recovered into bacteria were contaminants or unreplicated input DNA which remained in the cells. Plasmids grown in *dam*$^+$ bacteria such as HB101 or DH1 are methylated on every adenosine residue and are therefore sensitive to digestion by DpnI. The products of replication of that vector in a mammalian cell are not methylated on the adenosine residue and are therefore resistant to DpnI digestion. Thus, because in the above method of recovery the DNA is treated with DpnI prior to transformation of *E.coli* DH1 only DpnI resistant (*dam*$^-$) plasmids should be recovered. The results of the attempt to rescue plasmid from 18 different mouse cell lines is presented in Chapter 6.

2.13. Cloning of DNA fragments in Plasmids

2.13.1. Bacterial plasmid vectors

All the plasmids constructed during the course of this work were based on the plasmid vectors pAT153 (Twigg and Sherrat, 1980) and pBR328 (Soberon et al, 1980).

pAT153 is 3.66 kb in size and contains both ampicillin and tetracycline resistance markers. In the main the single HindIII and BamHI sites in the tetracycline gene were utilised.

pBR328 is a derivative of pBR325 (Bolivar, 1978). It contains chloramphenicol, ampicillin and tetracycline markers. The single PvuII site in the chloramphenicol marker, in addition to the tetracycline markers HindIII and BamHI sites were used for construction of vectors.

2.13.2. Ligations

Ligations were performed in sterile eppendorf tubes and were usually carried out using dephosphorylated (where applicable) linearised vector at concentrations of 2-5µg/ml depending on its size and hence its molar concentration. A 3:1 molar ratio of insert to vector was normally used.

The ligation reaction consisted of:-

Linearised vector DNA(2-5μg/ml), insert DNA at a 3-5 fold excess, 1/10th volume of ligation buffer (10x ligation buffer = 0.5M Tris pH7.5, 0.1M $MgCl_2$), 1mM dithiothreitol (freshly diluted from frozen 0.1M or 1M stock), 1mM rATP, T4 DNA ligase (Amersham), and made up to volume with sterile distilled H_2O). The reaction mix normally measured 10 or 20μl in volume with approximately 0.5 units of T4 ligase present. The reaction was incubated at 12°C overnight for ligation of sticky ends, when using linkers (next section) and for other blunt end reactions, incubations were at 4°C overnight.

All ligation reactions were normally controlled for by also ligating vector DNA alone and insert alone. The extent of ligation in all cases was checked on agarose gels before transforming competent bacterial cells with the recombinant vector.

2.13.3. Dephosphorylation of digested DNA

In most ligation reactions, where possible, the vector DNA was dephosphorylated at its 5' end prior to ligation. This dephosphorylation minimises recircularisation of the vector DNA since neither strand

of the duplex can reform a phosphodiester band. However, the insert DNA retaining its 5' terminal phophates can be ligated efficiently to the dephosphorylated plasmid to give an open circular molecule, thus giving efficient transformation of bacterical cells by mainly recombinant molecules. Dephosphorylation of vector DNA is of particular use when cloning into sites where no selectable marker system is available (that is if Amp^R, Tet^R, or Cam^R have previously been inactivated through insertion or deletion, as is often the case during the construction of vectors presented in this work).

The enzyme employed for dephosphorylation of the 5' ends of DNA molecules was calf intestinal alkaline phosphatase (CIAP). CIAP was purchased from Boehringer Mannheim in a lyophilised form. This solid CIAP was resuspended in dH_2O at a concentration of 2 units/ml and stored at -20^oC. Digested DNA was precipitated with ethanol and resuspended in the reaction buffer (50mM Tris HCl pH9.5, 0.1mM EDTA, 1mM spermidine). CIAP was added (1 unit/µg of DNA) and incubated at 37^oC for 30 minutes after which a second equal aliquot of CIAP was added and the reaction continued for a further 30 minutes. The reaction was terminated by heat inactivation at 70^oC for 10 minutes. The CIAP was removed extracting twice with phenol/chloroform/isoamyl alcohol followed by two extractions with chloroform/isoamyl alcohol.

2.13.4. Phosphorylation of synthetic linkers

Synthetic BamHI linkers d(CGGATCCG) were purchased from New England Biolabs. As synthetic linkers are not phosphorylated at their 5' ends, a 5' phosphate must be added to enable them to participate efficiently in ligation reactions. The method of Maniatis et al (1978, 1982) was used where the kinase and ligase reactions can be carried out sequentially in the same reaction mix. In this reaction γ-^{32}P labelled ATP is used as the phosphate donor and a proportion of the linker becomes labelled, ligation of the linkers can therefore be checked by incubating a proportion of them with ligase and analysing them on a polyacrylamide gel followed by autoradiography. Briefly, 1 OD_{260} unit of linkers (lyophilised) were dissolved in 100µl of TE (pH7.6) giving a concentration of 0.5mg/ml.

The kinase mix was as follows:- 4µl of linkers (~2µg) were incubated with 6.6pmoles (20µCi, lyophilised) of γ-^{32}P labelled ATP (Amersham International plc, sp act. >3000Ci/mmole), 1/10th volume of 10x linker-kinase buffer (10x linker- kinase buffer is 0.7M Tris-HCl (pH7.6),0.1mM $MgCl_2$, 50mM dithiothreitol), 1µl (10 units) of T4 polynucleotide kinase (Amersham International plc) and made up to 10µl with distilled H_2O. This mix was incubated at 37°C for 15 minutes and then the following mix was added in 10µl:1x linker kinase buffer, 1mM rATP and 10 units of T4

polynucleotide kinase. This was then incubated for a further 30 minutes at 37°C and then stored at -20°C in a lead pot.

To check that the kinased linkers could be ligated the following procedure was adopted. 1µl of the kinased linkers were added to a 1x ligation mix (see last section) with three units of ligase present in a 10µl mix. This was incubated at 4°C overnight.

The ligase was heat inactivated at 70°C for 10 mins. 5µl of the kinased, ligated linkers were transferred to a fresh tube and digested with 10 units of the appropriate restriction enzyme (BamHI) and its buffer in a 25µl reaction mix which was incubated at 37°C for 1-2 hours. The unligated, kinased linkers (2µl), ligated kinased linkers (5µl) and ligated, kinased, restricted linkers (5µl) were analysed by electrophoresis through an 8% polyacrylamide gel in 0.5 x TBE buffer (see section on polyacrylamide gels). The gel was run until the bromophenol blue had migrated 10cm into an 18cm gel. The gel was covered with cling film and exposed for autoradiography at -70°C.

For ligations of linkers to a blunt ended DNA fragment the linkers were added at a high concentration (20-50µg/ml), and a relatively low concentration (1-5µg/ml) of the fragment was used. The ligation mix was otherwise as described earlier except that a higher concentration

(3-5u/10µl) of ligase was used.

2.13.5. Construction and structure of plasmid vectors for transformation of mouse cell lines

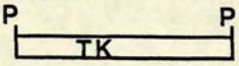
All vectors constructed were based on pAT153 or pBR328. The sequences inserted included Bovine papilloma virus-1 (BPV-1) and/or Herpes simplex virus thymidine kinase gene (HSV-TK; see Fig. 2.9) or amino glycoside phosphotransferase gene from bacterial transposon Tn5 (Neo^R) (see Fig. 2.9. SV2-Neo/TK-Neo). The following is a list of the plasmids used with a brief description of their construction, a diagrammatic representation of their construction is shown in Figures as indicated.

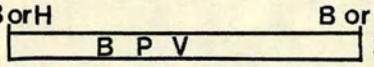
(a) pBR328/TK_2 - was constructed by C. Tyler Smith by the insertion of a 2kb PvuII fragment of HSV TK gene into the PuvII site of pBR328. The TK_2 refers to its orientation (see Fig. 2.3 pBR TK_2).

(b) pBR328/BPV/TK_2 - the 7.945kb HindIII linear of BPV-1 was cloned into the HindIII site of pBR328 TK_2. The orientation utilised has the BamHI site of BPV_1 closer to the EcoRI site of pBR328 (Fig. 2.3, pBPV/TK).

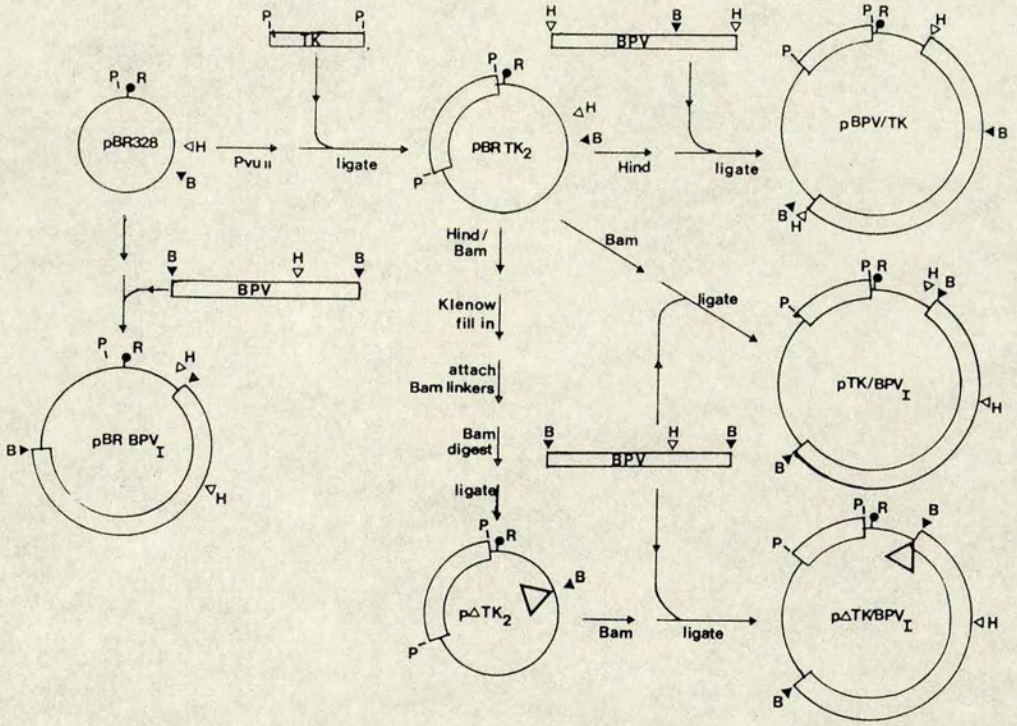
(c) pBR328/TK/BPV_I - the 7.945kb BamHI linear of BPV-1 was cloned into the BamHI site of pBR328 TK_2. The

FIGURE .2.3. Construction and simplified physical map of the vectors; pBR328, pBR328/TK_2 (pBR/TK_2), pBR328/BPV/TK_2 (pBPV/TK), pBR328/TK/BPV_I, (pTK/BPV_I), pBR328/BPV_I ($pBR\ BPV_I$), p△BR328/TK_2 ($p\triangle TK_2$), p△BR328/TK/BPV_I, ($p\triangle TK/BPV_I$).

The single lined regions represent pBR328 sequences. The triangle △ indicates deletion of the the pBR328 344bp Hind III/Bam HI fragment. The boxed region 

represents the 2040bp Pvu II fragment containing the HSV-TK gene. The boxed region 

represents the complete 7945bp Hind III or Bam HI cleaved BPV-1 genome. B = Bam HI sites, H = Hind III sites, R = Eco RI(1 of), P = Pvu II sites (2 of). For description of each vector and its contruction see Section 2.13.5.a to f.


TK
BPV
pBR328
Pvu II
ligate
pBR TK2
Hind
ligate
pBPV/TK
Hind/
Bam
Bam
Klenow
fill in
ligate
attach
Bam linkers
pTK/BPVI
BPV
pBR BPVI
Bam
digest
ligate
pΔTK2
Bam
ligate
pΔTK/BPVI

I refers to the orientation with respect of BPV, ulitilised (Fig. 2.3. pTK/BPV_I).

(d) pBR328/BPV_I - a BamHI linear of BPV-1 was inserted into the BamHI site of pBR328. The I refers to the orientation of the BPV insert and is identical to that in the previous construct (Fig 2.3. pBR BPV_I).

(e) pΔBR328/TK_2 - for reasons to be described elsewhere it seemed necessary to delete the 344bp HindIII/BamHI fragment from pBR328 to allow BPV-1 DNA containing vectors to replicate with plasmid sequences present in mammalian cells. To accomplish this pBR328 TK_2 was digested with HindIII and BamHI. The resulting large 6.562kb HindIII/BamHI fragment was isolated from an agarose gel, and the 5' overhanging ends filled in with DNA polymerase I Klenow fragment. Kinased BamHI linkers were then attached by ligation and sticky BamHI ends generated by BamHI digestion, this was then self ligated to give rise to pΔBR328/TK_2 from which the 344bp HindIII/BamHI fragment had been deleted (Fig. 2.3 and 2.4, pΔTK_2).

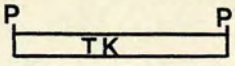
(f) pΔBR328/TK/BPV_I - the 7.945kb BamHI BPV-1 linear was inserted into the BamHI site of pΔBR328/TK_2. The resulting plasmid is identical to pBR328/TK/BPV_I except for the deletion of the 344bp HindIII/BamHI fragment (Fig. 2.3, pΔTK/BPV).

FIGURE 2.4. Construction and simplified physical map of the vectors; pΔBR328/TK (pΔTK_2), pΔBR328, pΔBR328/BPV_I (pΔBPV_I), pΔBR328/BPV/NEO_I (pΔBPV/NEO_I), p$\Delta IR/BPV_{II}$.

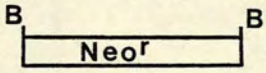
The single lined regions represent pBR328 sequences.

The triangle  indicates deletion of the the pBR328 344bp Hind III/Bam HI fragment.

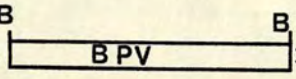
The larger triangle  indicates deletion of the pBR328 887bp Bcl I/BamHI fragment.

The boxed region 

represents the 2040bp Pvu II fragment containing the HSV-TK gene.

The boxed region 

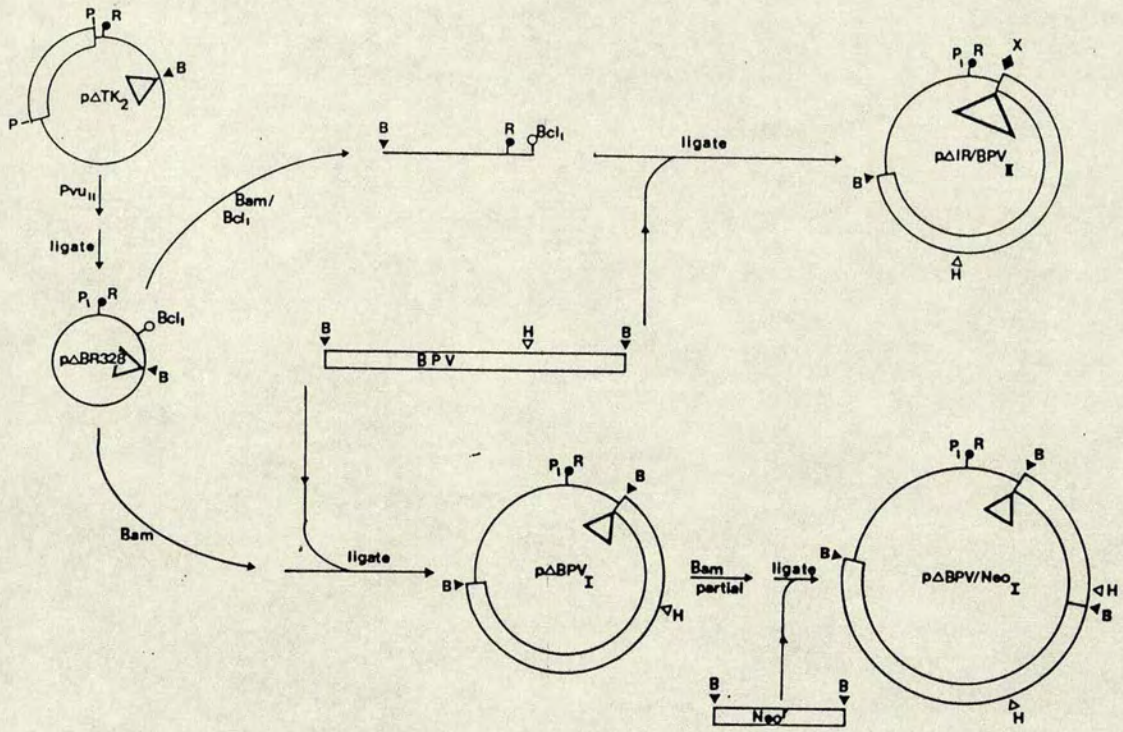
represents the 2160bp BamHI fragment containing the SV2.NEO transcription unit.

The boxed region 

represents the complete 7945bp Hind III or Bam HI cleaved BPV-1 genome.

B = Bam HI sites, H = Hind III sites, R = Eco RI(1 of), P = Pvu II sites (2 of), Bcl I = Bcl I site, X = Bcl I/ BamHI join with loss of site for either enzyme.

For description of each vector and its construction see Section 2.13.5.e to j.


pΔTK2
Pvu II
ligate
pΔBR328
Bcl I
Bam/
Bcl I
B
R
Bcl I
ligate
pΔIR/BPV I
X
H
B P V
Bam
ligate
pΔBPV I
Bam
partial
ligate
pΔBPV/Neo I
Neo r

(g) pΔBR328 - the 2kb PvuII HSV TK fragment was removed from pΔBR328/TK_2 by PvuII digestion and the resulting 4.562kb fragment self ligated. The plasmid is identical to pBR328, except that the 344bp HindIII/BamHI fragment has been deleted and it has lost resistance to tetracycline (Fig.2.4).

(h) pΔBR328/BPV_1 - the 7.94kb BamHI BPV-1 linear was inserted into the BamHI site of pBR328. The resulting plasmid pΔBPV_I is identical to pBR328 BPV_I except for the deletion of the 344bp HindIII/BamHI pBR328 fragment (Fig. 2.4, pΔBPV_I).

(i) pΔBR328/BPV_I/$NeO_{I,II,III,IV}$ - these four plasmids represent the four different orientations obtained, when a NeO^R gene (i.e. SV40 promoter and polyA addition signals, see Fig. 2.9) contained within a BamHI fragment of approximately 3.2kb from pBR322 Neo (see below) was inserted into pΔBR/BPV_I linearised by partial BamHI digestion. The resulting four plasmids are identical except for the orientiation of NeO^R with respect to the other plasmid sequences. Only orientation I is shown (Fig. 2.4, pΔBPV/Neo_I).

(j) pΔIR328/BPV_{II} - pBR328 is a derivative of pBR325 which was constructed by the addition of the chloramphenicol resistance gene to pBR322 (Bolivar 1978). Prentki et al (1981) found that both pBR328 and pBR325 carry a 482bp sequence at the end of the tetracycline

resistance gene which was duplicated in the opposite orientation between the chloramphenicol and tetracycline resistance genes in the construction of pBR325/pBR328.

Because it was thought that this inverted repeat (IR) may be inhibitory to the functioning of pBR328 based BPV vectors (further explanation elsewhere), it was removed from pΔBR328 by BamHI and BclI double digestion, followed by ligation of the 4.019kb pΔBR328 BamHI/BclI fragment to the BPV BamHI linear. The resulting circularised plasmid, pΔIR/BPV_{II}, contains BPV-1 cloned in orientation II, no recombinants with orientation I were recovered. 887bp of the original pBR328 sequence have been deleted, from the BamHI site to the BclI site. The construction of pΔIR/BPV_{II} required the ligation of a $BamHI_{\wedge}^{end}$ to a BclI sticky end with the resultant loss of a BamHI site and the BclI site (i.e. X on Fig. 2.4).

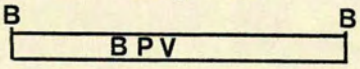
(k) pAT153/BPV_{I} - the 7.945kb BPV BamHI linear was inserted into the BamHI site of pAT153 (Fig.2.5) The recombinant tested, contained BPV in orientation I (Fig. 2.5 pAT/BPV_{I}).

(l) pΔAT153 - for the same reasons that the 344bp HindIII/BamHI fragment was deleted from pBR328 based vectors it was also deleted from pAT153. pAT153 was digested with HindIII and BamHI. The resulting 3.314kb fragment was purified from an agarose gel, and the 5'

FIGURE 2.5. Construction and simplified physical map of the vectors; pAT153, pAT153/BPV$_I$ (pAT BPV$_I$), pΔAT153, pΔAT153/BPV$_I$ (pΔAT BPV$_I$).

The single lined regions represent pAT153 sequences.

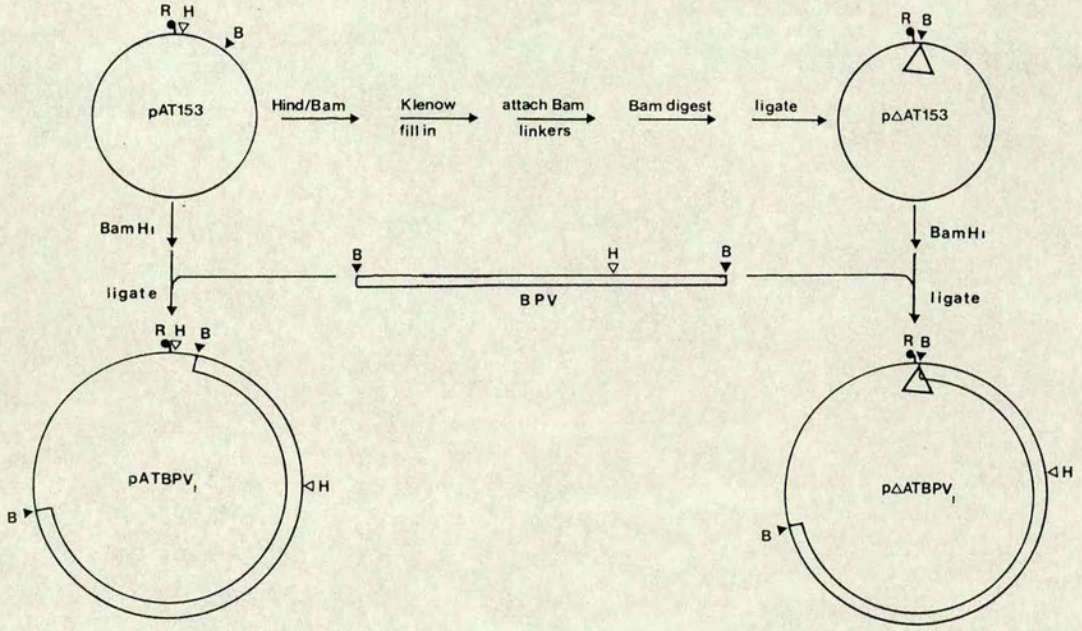
The triangle △ indicates deletion of the the pBR328 344bp Hind III/Bam HI fragment.

The boxed region 

represents the complete 7945bp Bam HI cleaved BPV-1 genome.

B = Bam HI sites, H = Hind III sites, R = Eco RI(1 of).

For description of each vector and its construction see Section 2.13.5.k to m.


R H
B
pAT153
Hind/Bam
Klenow
fill in
attach Bam
linkers
Bam digest
ligate
R B
pΔAT153
BamHI
ligate
B
H
B
BPV
BamHI
ligate
R H B
pATBPV1
H
B
R B
pΔATBPV1
H
B

overhanging ends filled in with DNA polymerase I Klenow fragment. Kinased BamHI linkers were then attached by ligation and sticky BamHI ends generated by BamHI digestion, this was then self-ligated to give pΔAT153 from which the 344bp HindIII/BamHI fragment has been deleted (Fig. 2.5).

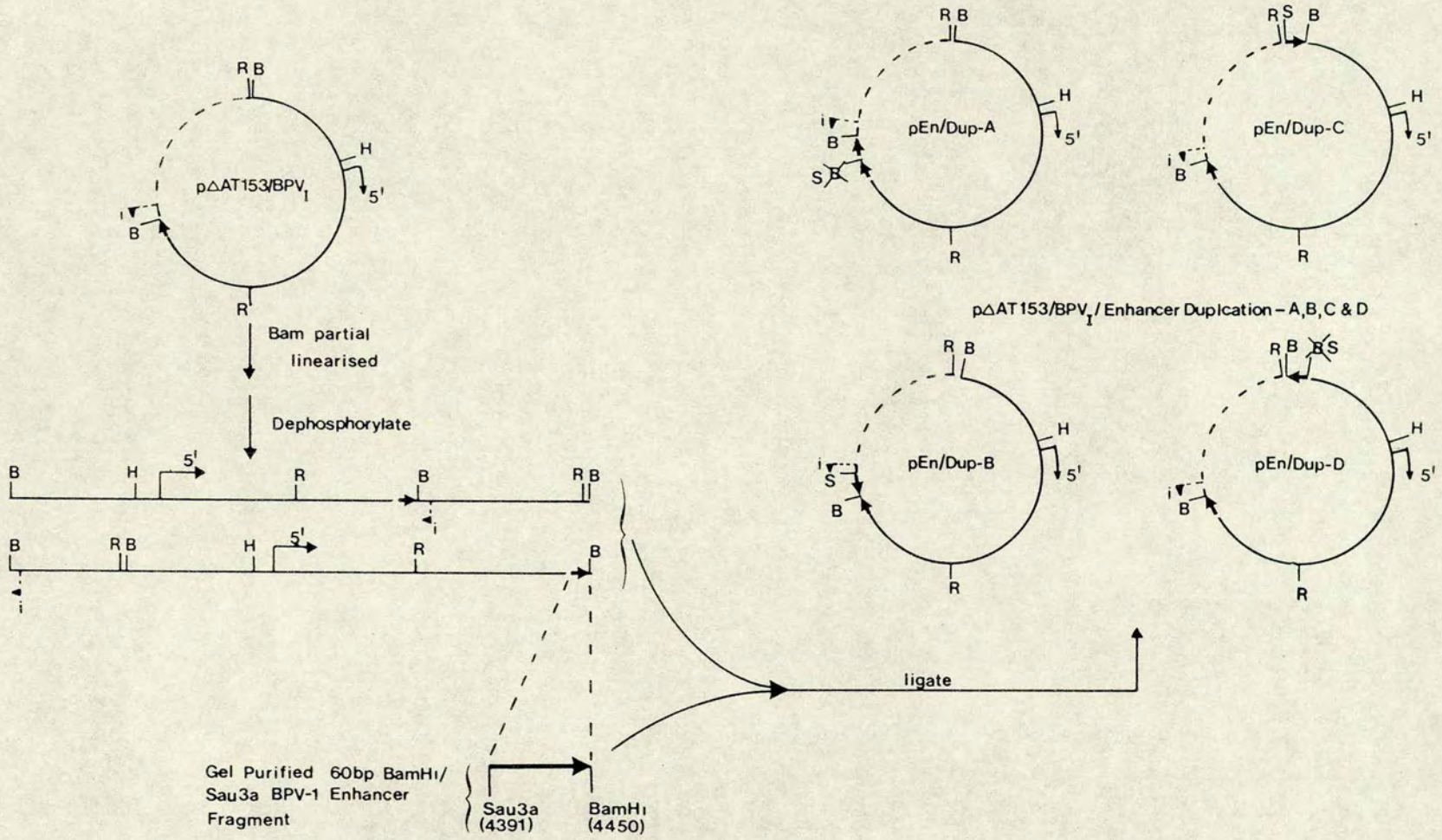
(m) pΔAT153/BPV_I - the 7.945kb BPV-1 BamHI linear was inserted into the newly formed BamHI site of pΔAT153. Both orientations of BPV-1 in pΔAT153 were isolated and tested for ability to replicate in C127 cells, only pΔAT153/BPV_I is shown (Fig. 2.5. pΔATBPV_I).

(n) pΔAT153/BPV_I/EN-DUP-A, B, C, & D: these four plasmids represent the four different orientations obtained when the BPV-1 60bp Sau3A/BamHI fragment which contains the BPV-1 enhancer was inserted into pΔAT153/BPV_I linearised by partial BamHI digestion. The four orientations are:- A, 60bp Sau3A/BamHI fragment inserted adjacent to the original BPV-1 enhancer as a direct repeat; B, the same fragment inserted adjacent to the original BPV-1 enhancer as an inverted repeat; C, the enhancer fragment inserted into the other BamHI site as a direct repeat, 3.3.kb away from the original enhancer; D, as in C, but inserted as an inverted repeat. (See Fig. 2.6, pEN-DUP, A, B, C, D). Orientation A was not isolated.

Some of the vectors used in this work were obtained

FIGURE 2.6. Construction and simplified physical map of the vectors; pΔAT153/BPV$_I$, pΔAT153/BPV$_I$/EN-DUP A, pΔAT153/BPV$_I$/EN-DUP B, pΔAT153/BPV$_I$/EN-DUP C, pΔAT153/BPV$_I$/EN-DUP D.

The broken lined regions represent pΔAT153 sequences.
The complete line region represents the complete 7945bp Bam HI cleaved BPV-1 genome.
The heavy arrow head (S ⟶ B) represents the 60bp Sau3A/BamHI fragment which contains the BPV-1 viral enhancer.
The symbol ⌐→ 5' indicates the 5' end of the BPV-1 early transcripts.
The symbol ⋮→ i indicates the position of and direction of transcription from the substitute promoter of pAT153.
B = Bam HI sites, H = Hind III sites, R = Eco RI(1 of), S = a Sau3A site.
For description of each vector and its construction see Section 2.13.5.m and n.


pΔAT153/BPV1
Bam partial linearised
Dephosphorylate
Gel Purified 60bp BamHI/ Sau3a BPV-1 Enhancer Fragment
Sau3a (4391)
BamHI (4450)
ligate
pEn/Dup-A
pEn/Dup-B
pEn/Dup-C
pEn/Dup-D
pΔAT153/BPV1 / Enhancer Duplication – A,B,C & D

as gifts from other laboratories.

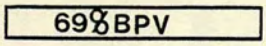
(o) pBrd BPV-β_1 - was obtained from D.DiMaio at Department of Biochemistry, Harvard University (DiMaio et al, 1982). pBrd BPV-β_1 - contains the 69% HindIII/BamHI transforming fragment of BPV1 (5.44kb) cloned between HindIII and BamHI sites of pBrd (a deletion derivative of pBr322 see Fig. 4.3). A 7.6kb HindIII fragment containing the Human β-globin gene was then inserted into the HindIII site. See figure 2.7.

(p) $pCGBPV_{9\Delta B5}$ - was obtained from P. Matthias, Institute of Cell and Tumor Biology, Heidelberg.

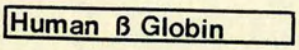
The construct $pCGBpV_{9\Delta B_5}$, is a derivative of $pCGBpV_9$ in that the BamHI site within the BPV-1 portion of the vector has been destroyed (Fig 2.8). $pCGBPV_{9\Delta B5}$ contains BPV_1 inserted as a HindIII linear into a plasmid containing a ColEI replication origin, a bacteriophage λ COS site and the Neo^R dominant selectable marker flanked by the HSV TK promoter region and polyadenylation signals (see Fig. 2.9 for TK-Neo transcription unit). The bacterial plasmid sequences are ColEI based.

(q) pBR322 Neo - was obtained from D. Canaani, Stanford University Medical Center (Canaani et al, 1982, Southern et al, 1982). pBR322Neo contains the Neo^R dominant selectable marker flanked by SV40 promoter and origin of replication DNA sequences and an SV40 3'

FIGURE 2.7. Simplified map of the vector pBRd BPVβ_1 as described by DiMaio et al (1982).



: Represents the 5437bp HindIII/BamHI (BPV69%T) fragment of the BPV-1 genome.



: Represents the 7600bp fragment of human genomic DNA which contains the human β-globin gene.

———————: Represents the pBRd plasmid sequences.

B = BamHI sites, H = HindIII sites, R = EcoRI sites.

For a description of the vector see Section 2.13.5.o.

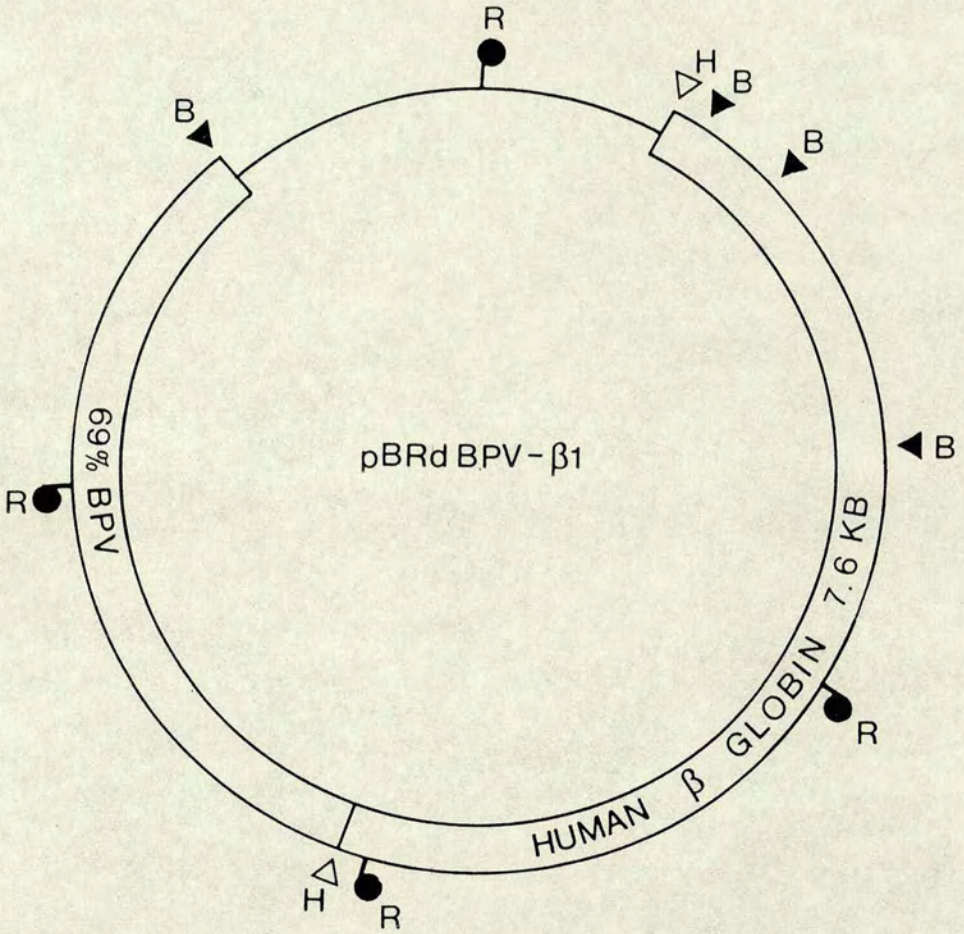

R
H
B
B
B
B
R
69% BPV
pBRd BPV - β1
HUMAN β GLOBIN 7.6 KB
R
H
R

FIGURE 2.8. Simplified map of the vector $pCGBPV_{9\Delta B5}$ obtained from P. Matthias.

———— : single lined region represents the plasmid sequences.

BPV-1 : open region represents a complete 7945bp cleaved BPV-1 genome in which the BamHI site has been destroyed (BamHI).

λcos : represents the COS site from bacteriophage λ.

TK-NEO : Hatched region represents the 2.0kb BamHI fragment containing the TK-NEO transcription unit (see FIG. 2.9).

Restriction endonuclease sites are as indicated. Arrows indicate the direction of transcription. For a description of this vector see Section 2.13.5.p.

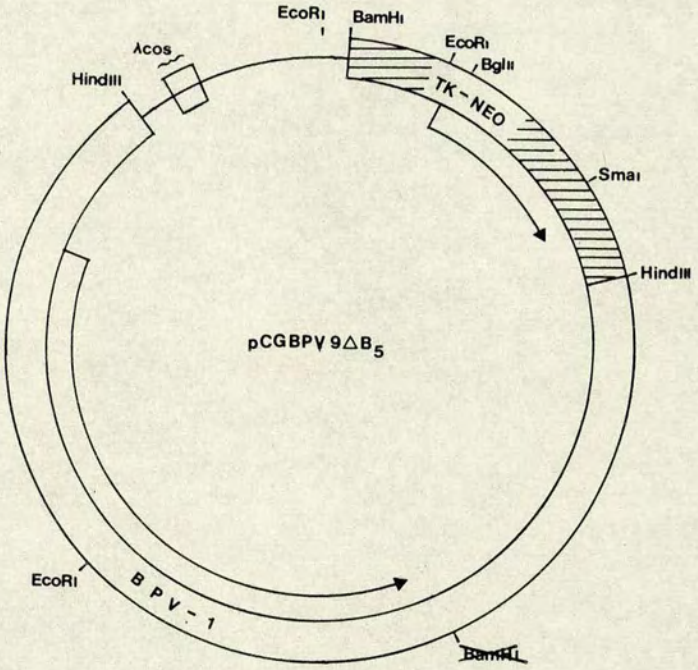
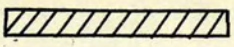
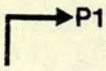

EcoRI
BamHI
λcos
EcoRI
BglII
HindIII
TK - NEO
SmaI
HindIII
pCGBPV 9△B5
EcoRI
BPV - 1
BamHI

FIGURE 2.9 Simplified physical map of the HSV-TK gene, and the TK-NEO and SV2.NEO transcription units.

: represents the transcriptional promoter unit from HSV-TK.

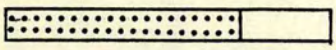


: represents the transcriptional polyadenylation site from HSV-TK.

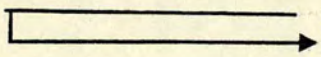


: represents the bacterial promoter P_1 from pBR322.

: represents the SV40 origin of replication and early promoter.

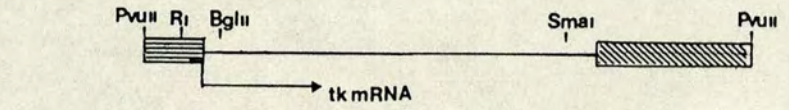


: represents SV40 3' intron and polyadenylation signals.



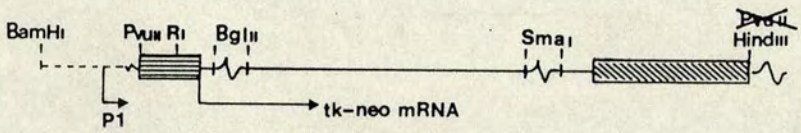
: represents the coding region of each gene with the direction of transcription indicated by the arrow.

Restriction endonuclease sites are as indicated.



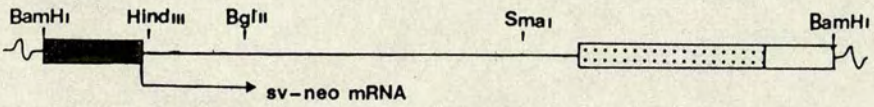
HSV TK

(2.040 Kb)



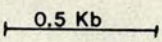
TK—NEO

(1.800 Kb Pvu II - Hind III)



SV_2—NEO

(2.610 Kb)

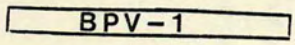


polyadenylation signal. The complete SV2-neo transcription unit is cloned as a BamHI insert in pBR322 (Fig.2.8 for SV2-neo unit).

(r) pML2d BPV-1 was a gift from P. Howley, NIH National Cancer Institute, Bethesda, Maryland). pML2d BPV-1 contains the BPV-1 BamHI linearised genome inserted into the BamHI site of pML2d (a deletion derivative of pBR322) (Fig. 2.10).

(s) pML2d/BPV/MMT-neo was also obtained from P. Howley and contains the Neo^R dominant selectable inserted into pML2d BPV-1 as a 4.0kb EcoRI/BamHI fragment (Fig. 2.11).

FIGURE 2.10. Simplified map of the vector pML2d/BPV-1 as described by Sarver et al (1982).



: represents the complete 7945bp BamHI cleaved BPV-1 genome.

———————— : represents the pML2d plasmid sequences, the d, indicates that the small 344bp HindIII/BamHI fragment has been deleted.

B = BamHI sites, H = HindIII sites, R = EcoRI sites.

For a description of the vector see Section 2.13.5.r.

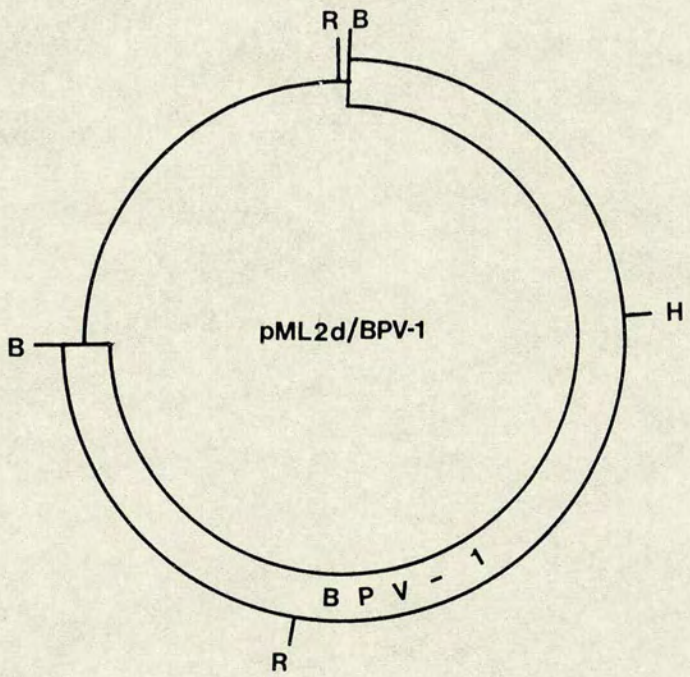
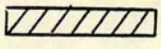
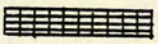
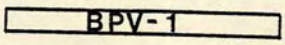

R
B
H
B
pML2d/BPV-1
B P V - 1
R

FIGURE 2.11. Simplified map of the vector pML2d/BPV/MMT-NEO as described by Law et al (1983).

 : represents the mouse metallothionein transcriptional promoter.

 : represents the bacterial neo gene from transposon Tn5.

 : represents the SV40 polyadenylation signal.



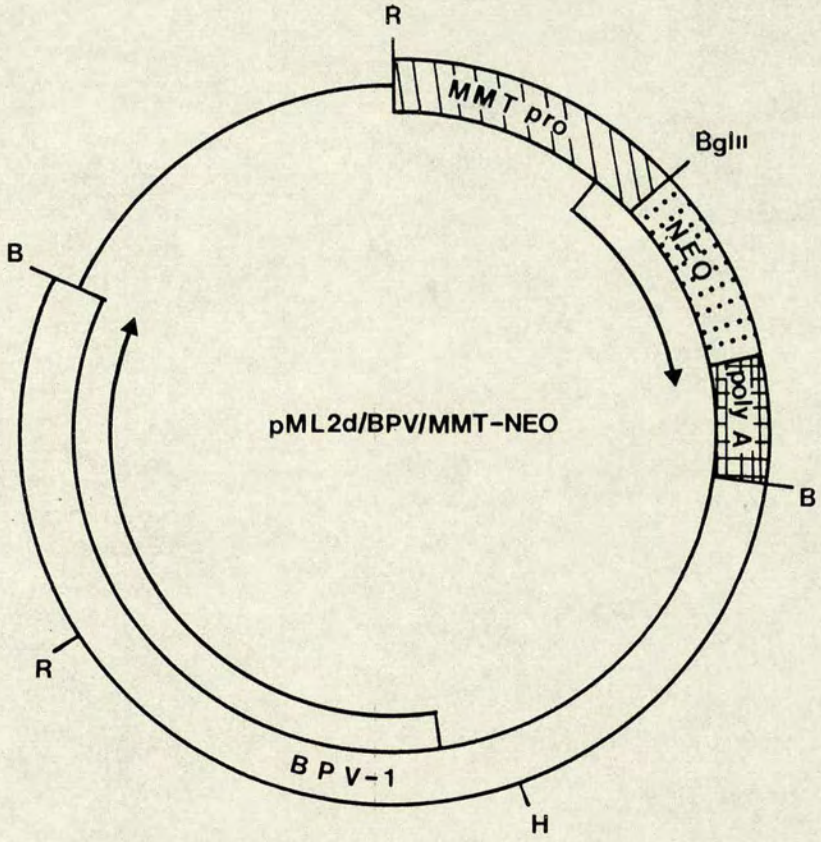
: represents the complete 7945bp BamHI cleaved BPV-1 genome.

——— : represents the pML2d plasmid sequences, the d, indicates that the small 344bp HindIII/BamHI fragment has been deleted.

Arrows indicate the direction of transcription.

B = BamHI sites, H = HindIII sites, R = EcoRI sites, BglII = BglII site (1 of).

For a description of the vector see Section 2.13.5.s.


R
MMT pro
BglII
NEO
poly A
B
B
pML2d/BPV/MMT-NEO
R
BPV-1
H

CHAPTER 3

EXTRACHROMOSOMAL REPLICATION OF BPV-1 DNA AND THE EFFECTS OF LINKAGE TO pBR328 AND THE HSV-TK GENE

3.1. Introduction

The autonomous replication of BPV-1 DNA as an episomal supercoiled circular molecule in mammalian cells was discussed in the Introduction. Transformation of cultured mammalian cells by BPV-1 was first demonstrated by Black *et al* in 1963. However, it was not until recently that the viral DNA was shown to replicate as a multicopy episomal supercoiled circular molecule in the nuclei of both bovine cells cultured from a BPV-1 induced bovine fibroma or mouse C127 cells transformed *in vitro* with either BPV-1 virus or DNA (Law *et al*, 1981; and Lancaster, 1981). Thus BPV-1 DNA with this transforming activity can be extracted directly from virus particles (Boiron *et al*, 1965) or can be derived from molecularly cloned BPV-1 DNA (Law *et al*, 1981).

The ability of the BPV-1 isolate (a gift from S. Campo, Glasgow) used in the studies presented here, to replicate as a supercoiled circular molecule in transformed cells was first tested. C127 cells were transformed either by the calcium phosphate precipitation method or by direct nuclear injection, using a complete BPV-1 genome DNA obtained by BamHI digestion of a plasmid ($p\Delta BR328/BPV_1$, Fig. 2.4) in which BPV-1 DNA is cloned as a 7.95kb BamHI linear fragment. This transformation was designed so as to repeat closely that presented by Law *et al* (1981). These workers cloned the BPV-1 genome as a BamHI linear fragment in the plasmid pBR322, but

separated the BPV-1 sequences from the bacterial sequences by cleavage with BamHI. The whole digestion mix (BPV-1 100% + pBR322) was then used to transform C127 cells.

The results presented in this Chapter show that the isolate of BPV-1 used throughout the work described in this thesis was capable of recircularising and replicating as an extrachromosomal supercoiled circular molecule within C127 cells, but that often a large quantity of high molecular weight BPV-1 molecules were also detected. Thus, this BPV-1 isolate appeared to replicate in the expected manner, in agreement with the results of Law et al (1981), and therefore vectors based on it linked to bacterial plasmid sequences and the Herpes simplex virus - thymidine kinase gene (HSV- TK) were constructed. Their replication was analysed in C127 cells when foci were selected or in Ltap207 cells when TK^+ transformants were selected. The analysis of total cellular DNA isolated from such tranformants is presented in this Chapter.

3.2. Analysis of cellular DNA from cell lines transformed with pΔBR328/BPV$_I$ digested with BamHI

Total cellular DNA was prepared from C127 mouse fibroblast cell lines transformed with BamHI cleaved pΔBR328/BPV-1 either by the calcium phosphate

precipitation or by the microinjection method. The BPV-1 sequences present in the DNA of a transformed cell were analysed by blotting experiments. Firstly, mildly sheared samples of the 14 cell lines were run on 0.6% agarose gels and probed with radioactively labelled BPV-1 (gel purified BamHI fragment). The result (Fig.3.1.A) shows that in the absence of digestion, DNA homologous to BPV-1 was present not only as a monomeric, episomal supercoiled circular form, but also as four other larger discrete sized forms; i.e. specific bands were detected, which could represent concatemeric (dimer, trimer etc.) or catenated (interlocked monomeric circular) forms. In addition to these five specific bands which were visible in all samples, a large proportion of the BPV-1 DNA was detected as a slow migrating species, which did not resolve into specific bands. Such a species had previously been reported by Law et al (1981) who suggested that it consisted of interlocked monomeric circular forms since they claimed it could be converted to monomeric, linear/open circular/supercoiled forms by mild S1 nuclease digestion. The structure of this high molecular weight species of BPV-1 DNA is of paramount importance since it could also represent integrated forms of the viral DNA. To understand more fully the nature of this high molecular weight species a thorough analysis of one of these cell lines, Clone 2 in Fig.3.1.A, will be presented in Chapter 7.

Further analyses of the DNA from these cell lines by

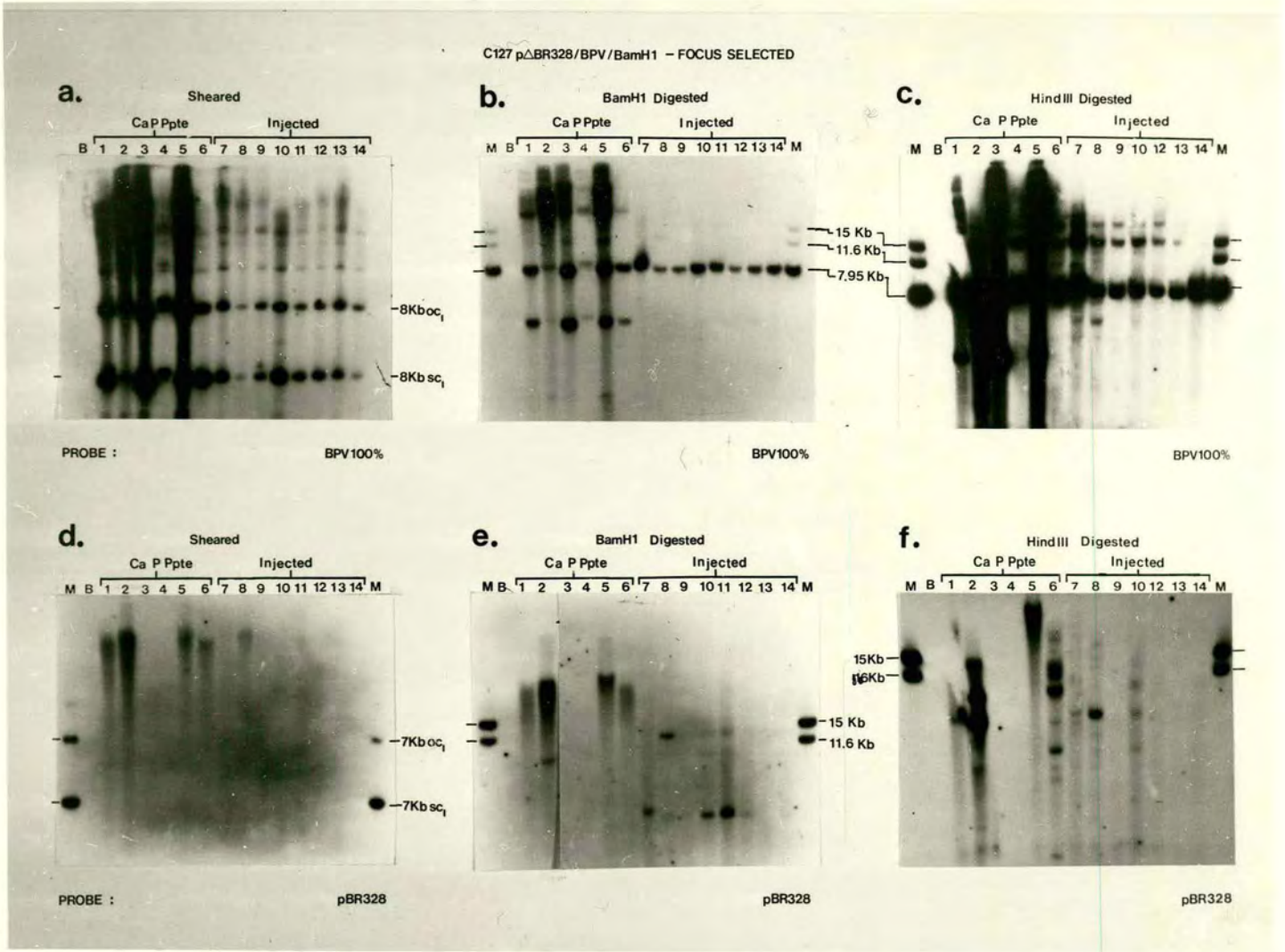
FIGURE 3.1. BamHI cleaved pΔBR328/BPV_I DNA in focus selected C127 cell lines.

Cellular DNA (5µg) was sheared by passage through a 25 gauge syringe (a & d) or digested with a fivefold excess of BamHI (b & e) or HindIII (c & f). Samples were electrophoresed through 0.7% agarose gels, transferred to nitrocellulose and hybridised to a nick translated probe, BPV-1 in a, b & c, pBR328 in d, e & f.

Samples:- B; Ltap207 non transformed total cellular DNA. M; Markers, size as indicated on each autorad. contain approx. 10 copy equivalents of BPV-1 or BPV-1 containing plasmids in 5µg of Ltap207 DNA. 1; DNA extracted from C127 pΔBR328/BPV_I/BamHI Clone 1. 2; Clone 2. 3; Clone 3. 4; Clone 4. 5; Clone 5. 6; Clone 6. 7; Ij 1a. 8; Ij 1b. 9; Ij 2a. 10; Ij 2b. 11; Ij 3a. 12; Ij 14a. 13; Ij 14b. 14; Ij 18a.

Samples 1-6 were derived by calcium phosphate precipitation.

Samples 7-14 were derived by microinjection.


C127 pΔBR328/BPV/BamH1 – FOCUS SELECTED
a.
Sheared
Ca P Ppte
Injected
B 1 2 3 4 5 6 7 8 9 10 11 12 13 14
8Kb oc
8Kb sc
PROBE :
BPV100%
b.
BamH1 Digested
Ca P Ppte
Injected
M B 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
15 Kb
11.6 Kb
7.95 Kb
BPV100%
c.
HindIII Digested
Ca P Ppte
Injected
M B 1 2 3 4 5 6 7 8 9 10 12 13 14 M
BPV100%
d.
Sheared
Ca P Ppte
Injected
M B 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
7Kb oc
7Kb sc
PROBE :
pBR328
e.
BamH1 Digested
Ca P Ppte
Injected
M B 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
15 Kb
11.6 Kb
pBR328
f.
HindIII Digested
Ca P Ppte
Injected
M B 1 2 3 4 5 6 7 8 9 10 12 13 14 M
15Kb
pBR328

cleavage with BamHI and HindIII, enzymes which cut BPV-1 DNA at only one site (Fig. 3.1,B and C)) revealed that the majority of BPV-1 DNA in transformants derived by microinjection is converted to a fragment which co-migrates with the full sized linear BPV-1 DNA marker (7.95kb). Digestion of the DNA from calcium phosphate precipitate derived transformants with BamHI leaves a large amount of undigested supercoiled, open circular and higher order forms. This suggests that, on entering the cell cytoplasm, a proportion of the BPV-1 BamHI linear molecules were damaged at their ends so that upon recircularisation the BamHI site was destroyed, leaving a mixture of BamHI digestible and undigestible molecules in each transformant. In the microinjected cell lines no supercoiled or open circular forms remain visible following BamHI digestion, suggesting that all the episomal molecules had retained the BamHI site. This is probably a consequence of protection from degradative enzymes by direct nuclear injection, since most nucleases reside in lysosomes in the cell cytoplasm through which the DNA-precipitate complex may pass (Luthman and Magnusson, 1983).

In the BamHI digested tracks of the DNA from micro-injected cell lines, other faint bands are visible at ~12kb (lane 8 and 10) and ~15kb (lanes 10, 11, 12, 14). These possibly represent the 'religation' of linear BPV-1 to either a second BamHI linear BPV-1 or a pΔBR328 linear with loss of the intervening BamHI site giving

rise to bands of nominally 16kb and 12.6kb, respectively. This is further supported by the presence of other complex unexpected bands in the HindIII digests, i.e. major band of 8kb in each case with minor bands visible at ~6kb, 9kb, 12kb, 15kb and 20kb. Some of these bands are present at the level of ~1 copy/cell and, could, therefore, represent junction fragments between BPV-1 and a mouse chromosomal sequences at putative sites of integration. Alternatively they may represent minor rearranged extrachromosomal forms.

When sheared samples of these 14 cell lines were probed with nick translated pΔBR328 (Fig. 3.1.D), so as to detect any cotransforming plasmid sequences, high molecular weight material was seen to hybridise to the probe in many of the cell lines. Since no specific bands and only a high molecular weight smear hybridised to the plasmid probe, it is likely that all the plasmid sequences are present in a high molecular weight complex which may be integrated, and not as detectable episomal supercoiled or open circular forms. On probing BamHI digests of these cell lines with pBR328 (Fig. 3.1.E), a band corresponding to the size of linear pΔBR328 (4.6kb) could be detected in 7 out of 8 of the injection derived cell lines. Bands of other sizes could also be detected, suggesting linkage of pΔBR328 to BPV-1 with loss of the intervening BamHI site (i.e. 12kb band in lane 8, Figure 3.1.E, is equivalent to that in Fig. 3.1.B). Rehybridisation of the filter in Fig. 3.1.C. with pΔBR328 also revealed the

presence of pBR328 sequences as specific bands in each cell line (Fig. 3.1.F.). In this case the HindIII digests contain multiple bands, some of which may have resulted from linkage of BPV-1 to plasmid and others of which could conceivably have arisen by the integration of plasmid sequences into a host cell chromosome, generating unusual sized junction fragments in the process.

Thus, the results show, in agreement with those of Law et al (1981), that the introduction of BamHI linearised BPV-1 DNA into mouse C127 cells causes the formation of foci upon transformation. Probing of purified DNA from these foci with BPV-1 shows that viral DNA is present as monomer supercoiled and open circular forms, as well as in other higher molecular weight structures. Also in agreement is the fact that, in those cell lines derived by calcium phosphate precipitation, many of the replicating molecules had lost the BamHI site which had been at the cohesive ends of the introduced molecule. In contrast to this none of the cell lines derived by microinjection were seen to lose the BamHI site, and it is therefore apparent that microinjection of DNA avoids the degradative processes encountered in the calcium phosphate precipitation technique (Luthmann and Magnusson, 1983). This conclusion is also supported by the observations of Folger et al (1982_2).

3.3. Introduction of a BPV-1 vector containing the HSV/TK selectable marker into Ltap207 cells.

The results presented so far suggested that this BPV-1 DNA can replicate as an extrachromosomal form in mouse C127 cells. Bearing in mind that it could not be established whether or not a proportion of the higher molecular weight species represented forms which were integrated into a host chromosome, a vector was constructed in which the complete BPV-1 genome (HindIII fragment) and the Herpes Simplex Virus thymidine kinase gene (HSV-TK PuvII fragment, Fig. 2.9) were cloned into pBR328. This vector pBR328/BPV/TK2 (constructed by C. Tyler-Smith, Fig. 2.3) was introduced into the Ltap207 TK^- cell line and TK^+ colonies selected in HAT medium. Total cellular DNA was purified from six TK^+ transformants following calcium phosphate transformation with the plasmid pBR328/BPV/TK2 prepared after growth in *E.coli* HB101 *dam*$^+$, and another five TK^+ transformants similarly derived from plasmid grown in *E.coli* CSH26, a *dam*$^-$ strain. Both *dam*$^+$ and *dam*$^-$ forms of the vector DNA were used because Calos *et al* (1983) and Lusky *et al* (1983), had suggested that a BPV-1 vector containing the HSV-TK gene would only replicate as an intact episome in rodent cells if the plasmid DNA had been propagated in a *dam*$^-$ *E.coli* strain.

The DNA from these 11 TK^+ cell lines was

analysed by mild shearing of the DNA and blot hybridisation using nick translated pBR328/BPV/TK2 as a probe. In none of the TK^+ cell lines was DNA detected which migrated at a position similar to that of the supercoiled or open circular input plasmid (Fig. 3.2.A). All cell lines, except <u>dam</u>$^-$ clone 3 lane 9, contained hybridising material which ran only at the position of high molecular weight DNA (that is greater than 30kb).

After HpaI restriction endonuclease digestion (there is only one HpaI cleavage site in the vector) all cell lines were seen to have different patterns of hybridisation. A band of ~15kb was seen in some of the cell lines, but other faint bands were also visible in these DNA samples (Fig. 3.2.B). Similar results were also found upon digestion with KpnI (one cleavage site - results not shown). These results suggest that in each cell line (except <u>dam</u>$^-$Cl 3) vector DNA had probably integrated as head to tail tandem arrays of intact, rearranged and/or deleted plasmids which is consistent with the behaviour of other TK vectors (J.P. Simons, 1985 and C. Tyler-Smith, unpublished work). Analysis of the DNA of Ltap207 TK^+ pBPV/TK2 <u>dam</u>$^-$Cl 3 (i.e. Track 9) by shearing showed the presence of two specific low molecular weight bands, suggesting that these are episomal forms of the input vector. However, because they ran with a mobility not expected for intact input plasmid, it is presumed that they represent rearranged

FIGURE 3.2. pBR328/BPV1/TK_2 DNA in TK^+Ltap207 cell lines.

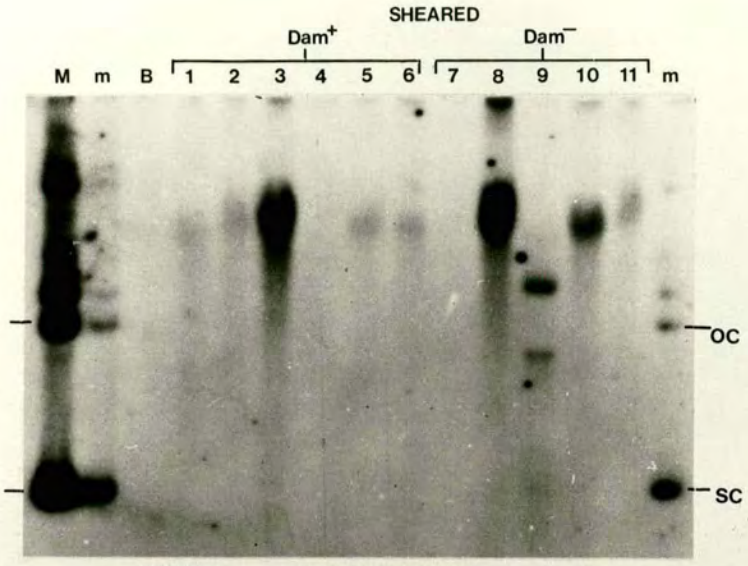
Cellular DNA (5µg) was sheared by passage through a 25 gauge syringe (a) or digested with a fivefold excess of HpaI (b). Samples were electrophoresed through 0.65% and 0.5% agarose gels respectively, transferred to nitrocellulose and hybridised to a nick translated probe, pBR328/BPV-1/TK_2 in a, BPV-1 in b.

Samples:- M (a); Size marker for input form of pBR328/BPV-1/TK_2 at 50 copy equivalents in 5µg in Ltap207 DNA.
m (a); as in M (a) but with a 10 copy equivalent. m (b); 10 copy equivalent of linearised BPV-1, or plasmids containing BPV-1 (sizes as indicated) in 5µg of Ltap207 DNA. B; Ltap207 non-transformed total cellular DNA. 1; DNA extracted from Ltap207 TK^+ pBR328/BPV1/TK_2 dam^+ Clone 1, 2; Clone 2. 3; Clone 3. 4; Clone 4. 5; Clone 5. 6; Clone 6. 7; DNA extracted from Ltap207 TK^+ pBR328/BPV1/TK_2 dam^- Clone 1, 8; Clone 2. 9; Clone 3. 10; Clone 5. 11; Clone 6.

Samples 1-6 were transformed with HB101 dam^+ prepared pBR328/BPV-1/TK_2.
Samples 7-11 were transformed with CSH26 dam^- prepared pBR328/BPV-1/TK_2.

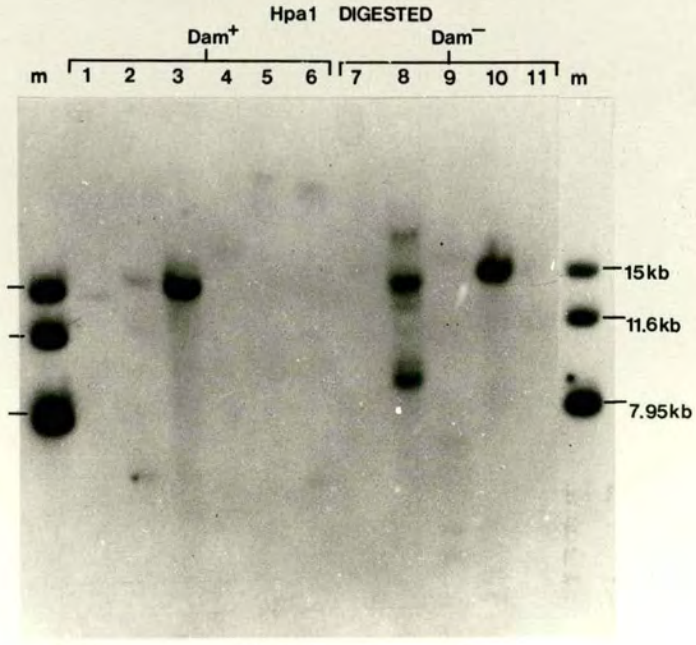
Ltap207 pBR328/BPV-1/TK_2 :- TK SELECTED.

a.



PROBE : pBR328/BPV/TK

b.



PROBE : BPV 100%

and/or deleted forms. The possibility that these bands resulted from contamination with exogeneously added plasmid was not investigated. An attempt had previously been made (C. Tyler-Smith, unpublished work) to recover pBR328/BPV/TK2 DNA from low molecular weight DNA prepared from similarly transformed Ltap207 cells, following transformation into E. coli HB101 and selection for ampicillin resistant colonies. Amp^R transformants were obtained at a very low frequency, which suggested that the recovered molecules were present in the mammalian cell cultures at a frequency of about 1 per 50 cells. Analysis of these recovered plasmids showed that they contained large stretches of scrambled vector sequences. When a second round of transformations were carried out using the recovered, scrambled vector, only re-rearranged forms were recovered from the secondary transformants.

3.4. Introduction of pBR328/BPV/TK2 into C127 cells by Focus Selection

Ltap207 TK^- cells are already morphologically transformed and, therefore, unlike C127 cells do not grow as a contact inhibited monolayer. It is conceivable that the pBR328/BPV/TK2 vector did not replicate as an unrearranged episomal circular molecule in the TK^+ transformed Ltap207 cells because of the absence of selection for the BPV-1 transformation function and,

therefore, its expression. To test this possibility the vector pBR328/BPV/TK2 was also introduced into C127 cells by the calcium phosphate precipitation procedure. Foci were picked and expanded into cell lines, total cellular DNA was then examined by DNA shearing and hybridisation using the complete vector as a probe. Fig. 3.3.A shows that in the 10 cell lines examined only one cell line (clone 1) contained a band which comigrated with the monomer supercoiled circular form of the input plasmid DNA. The other 9 cell lines contained bands which migrated at a position faster than the input plasmid and must, therefore, have undergone deletion of some of the vector sequences.

To investigate whether any particular vector sequences were deleted in common amongst the 10 cell lines, sheared samples of the DNA from these were run on 5 agarose gels in parallel, transferred to nitrocellulose and probed with 5 separate probes. These probes were (see Fig. 3.3) A, pBR328/BPV/TK2 (referred to above); B, BPV69%T HindIII/BamHI; C, BPV31%NT HindIII/BamHI; D, pΔBR328; E, HSV/TK PvuII fragment. The results suggested that, as expected, the BPV69%T fragment necessary for focus formation (transformation-T) was unaffected by deletions. The BPV31%NT non-transforming (NT) fragment seemed to be present in the same relative amounts in all cell lines, except the cell line shown in lane 3 which contains the smallest episomal form which would seem to have lost a large proportion of the

FIGURE 3.3. pBR328/BPV-I/TK_2 DNA in C127 focus selected cell lines.

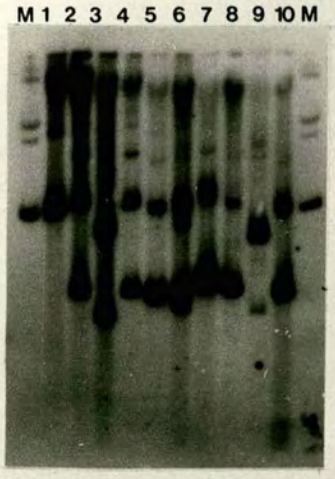
Cellular DNA (5µg) was sheared by passage through a 25 gauge syringe (A-E)or digested with a fivefold excess of HpaI (F). Samples were electrophoresed through 0.65% agarose gels, transferred to nitrocellulose and hybridised to a nick translated probe, pBR328/BPV-1/TK_2 in A, BPV69%T in B, BPV31%NT in C, p BR328 in D, HSV-TK PvuII in E, and BPV-1 in F. All these cell lines were derived by calcium phosphate precipitation.

Samples:- M (A-E);10 copy equivalents of input plasmid pBR328/BPV-1/TK_2 in 5µg in Ltap207 DNA. M (F); 10 copy equivalent of linearised BPV-1, or plasmids containing BPV-1 (sizes 15kb and 11.6kb) in 5µg of Ltap207 DNA. 1; DNA extracted from C127 pBR328/BPV-I/TK_2 dam$^+$
Clone 1 pl 1 rc, 2; Clone 2 pl 1 rc. 3; Clone 2 pl 2 rc. 4; Clone 3 pl 1 pool. 5; Clone 5 pl 2 rc. 6; Clone 5 pl 2 pool. 7; Clone 3 pl 2 rc. 8; DNA extracted from C127 pBR328/BPV-I/TK_2 dam$^-$ Clone 1 pl 1 rc. 9; Clone 4 pl 1 rc. 10; Clone 5 pl 1 rc.

Samples 1-7 were transformed with HB101 dam$^+$ prepared pBR328/BPV-1/TK_2.
Samples 8-10 were transformed with CSH26 dam$^-$ prepared pBR328/BPV-1/TK_2.

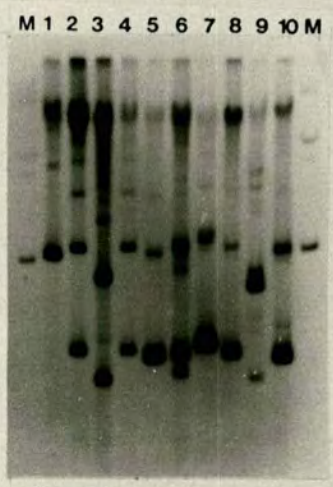
C127 pBR328/BPV1/TK2 FOCUS SELECTED

A



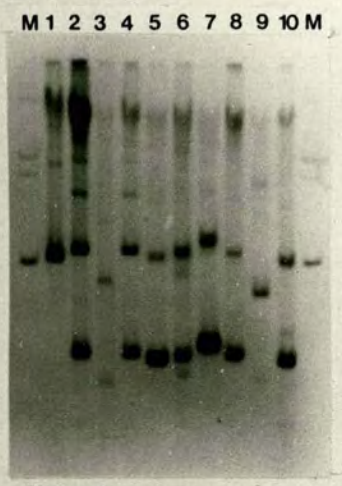
Sheared
Probe : pBr328BPV/TK

B



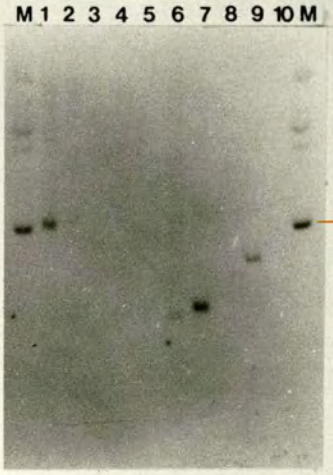
Sheared
Probe : BPV 69%T

C



Sheared
Probe : BPV 31%NT

D



Sheared
Probe : pΔBr328

E

Sheared
Probe : TK Pvu II

F

Hpa I Digested
Probe : BPV 100%

BPV31%NT fragment on comparison with the band intensity in neighbouring lanes in Fig. 3.3.C with Fig. 3.3.B. On probing with the plasmid pΔBR328 (Fig. 3.3.D) a dramatic reduction in the amount of hybridisation in many cell lines was seen, suggesting that most or all of the plasmid sequences were missing, through deletion.

When probed with the HSV-TK PuvII fragment (Fig.3.3.E) only two lanes, 5 and 6, (which are a cell line in lane 6 and its subclone, in lane 5) showed any homology to the HSV-TK probe, suggesting that in all the other cell lines most or all of the TK sequences had been deleted.

Digestion of the same cell line DNAs with the enzyme HpaI (which cuts once within the BPV69%T of pBR328/BPV/TK2) and probing with BPV-1 (Fig. 3.3.F.) revealed that all of the BPV-1 containing DNA material is converted to a size which ranges from ~6kb to 9kb in all cell lines apart from one; the exception in lane 1. It is of interest at this stage to note that the complete BPV-1 genome is 7.95kb in size, but that the necessary BPV69%T fragment which retains the replication and transformation functions is only 5.5kb in size (Lowy et al, 1980, Law et al, 1981). It is possible that the major form of approximately 15kb in the cell line in lane 1 consists of a deleted dimer of some sort in which only one HpaI site resides. KpnI (another enzyme which cuts once within pBR328/BPV-1/TK2) digestion of this cell line DNA

resolves the BPV-1 containing sequences into two major bands of similar sizes (approximately 7 and 8kb, results not shown) which suggests that dimerisation of a deleted form of the vector had occurred (data not shown).

The small fragment of approximately 2kb seen at the bottom of the HpaI digestion of this cell line DNA may represent a junction fragment from an integrated form, but more likely was generated upon cleavage of the rearranged dimeric extrachromosomal form. In the remaining DNA samples other bands of weak intensity are also visible. These could represent junction fragments generated upon digestion with HpaI of copies integrated in the chromosomal DNA, but it is just as likely that they result from the presence of other minor rearranged extrachromosomal forms. This is the case in lane 6 (Fig. 3.3F) where clearly the two bands created by HpaI digestion were generated from the major and minor supercoiled monomer seen in Fig. 3.3.A,B & C. Therefore although the possibility of integration of the vector DNA in these focus selected lines still exists, the analyses presented do not conclusively demonstrate that such an event occurred.

3.5. Discussion

The results presented above clearly indicate that the BPV-1 isolate used in these studies behaves in a

manner very similar to that used by Law et al (1981). However, here the analysis has been extended by introducing the pΔBR328/BPV$_I$/BamHI cleaved DNA by direct nuclear injection in addition to the calcium phosphate precipitation technique. The precipitation method used here differed from that of Law et al (1981) in that no carrier DNA was included in these experiments during the formation of the precipitate, and, therefore, a much larger quantity of the pΔBR328/BPV$_I$/BamHI cleaved DNA was required (i.e. 20μg/ml of precipitate). This modification has the advantage in that only the desired and well defined sequences, (i.e. plasmid and BPV-1) gain access to the cell and no other uncharacterised DNA is included. The apparent efficiency of transformation, as measured by the number of foci/μg of BPV-1 DNA, obtained in the absence of carrier is seen to be less than that obtained by carrier mediated transformation presumably because of the large excess of plasmid DNA required for the formation of the precipitate. However, when the number of cells per μg of added BPV DNA is taken into account the transformatin efficiencies in the presence or absence of carrier DNA are comparable. Using the 'carrier free' type of transformation technique an average of 12 foci per μg of BPV-1 DNA per 5.0×10^4 cells were obtained when BamHI cleaved pBR328/BPV-1 DNA was precipitated onto mouse C127 fibroblast (see Table 2.1 and Section 2.1.2.k). This is comparable with the results of Lowy et al, 1980, Dimaio et al, 1982 and others who have reported frequencies of approximately 200-

250 foci per µg of BPV-1 DNA per 7.5 x 10^5 C127 cells in similar experiments where 2.5µg of the BPV-1 DNA were precipitated in the presence of 25µg of calf thymus DNA as carrier. Thus in both types of transformation, frequencies approaching a maximum are attained. Kushner et al (1982) have reported transformation frequencies of the order of 10-25 foci per µg of transfected recombinant BPV-1 DNA per 1 x 10^6 cells when using the 'carrier free' transformation method, these frequencies were probably lower than those obtained here because the amount of BPV-1 DNA applied per cell was well below that required to obtain a maximum transformation frequency.

The analysis carried out here extends the studies of Law et al (1981) in that a total of 14 cell lines were examined, 6 derived by precipitation and 8 by injection and because the possibility of cotransferred bacterial plasmid sequences (pΔBR328) present in the introduced DNA was also investigated. The results clearly indicate the presence of these plasmid sequences in 10 out of the 14 cell lines examined. Digestion with BamHI and probing with pBR328 or BPV-1 suggest the linkage of both BPV and plasmid sequences in tandem arrays, but whether these structures are integrated into the host genome is not known. However, the fact that the plasmid sequences were not detected as specific bands in the undigested sheared samples (Fig. 3.1.D) suggests they are part of a very large structure which is randomly

fragmented upon mild shearing.

HindIII digestion reveals the presence of other bands besides the expected 7.95kb band of intact linear BPV-1 (Fig.3.1.C). Many of these were also detected when the same filter was probed with pBR328 (Fig.3.1.F), suggesting again the linkage of pΔBR328 to BPV-1 sequences on entry to the cell. However, many other bands in these HindIII (which does not cut within pΔBR328) digested DNA samples were also detected which were not found when only BPV-1 was used as a probe (Fig. 3.3.C and F). This suggests that in many cases single or multiple copies of the plasmid sequences alone had integrated at several chromosomal positions. The possibility also exists that the plasmid sequences had become linked to BPV-1 and then integrated into the host chromosome. However, it cannot as yet be concluded that any of the BPV-1 sequences are integrated into a host chromosome in any of these cell lines, and a more detailed analysis of this possibility will be presented in chapter 7.

The fact that plasmid sequences were so readily detected and that their presence may have resulted in the integration of viral DNA into the host genome, is of great importance if the unique property of BPV-1 to replicate as a circular molecule in mammalian cells is to be exploited for the construction of cloning/expression vectors based on this virus. In contrast to these

results, Law et al (1981) did not detect any band other than the 7.95kb linear form of BPV-1 on hybridisation of either BamHI or HindIII cleaved cell line DNA with a nick translated BPV-1 probe. They indicated in reconstruction experiments that the sensitivity of their analysis was capable of detecting 0.1-0.2 viral genomes per diploid cell transformed with BPV. Their copy control marker consisted of a 12.3kb plasmid containing BPV-1, a single copy of which could easily be detected in the presence of 38 copies of the viral 7.95kb fragment released by BamHI digestion of the DNA. The 0.1/0.2 copies were only detected when the relative amount of endogenous BPV-1 was also reduced to 3.8/7.6 copies. If they had been present, any other homolgous single copy bands of less than 8kb would not have been detected due to the background smearing created by digestion of the 38 copies of viral DNA present in the cell (see Law et al, 1981, Fig. 4). It is therefore possible that bands other than the 7.95kb linear BPV-1 fragment were present in the transformants of Law et al (1981) but remained undetected. It is also not known whether or not any linearised bacterial plasmid sequences (pBR322) present in the DNA/calcium co-precipitate of Law et al (1981) were taken up by their cell lines. It would be more likely that any BPV-1 DNA molecules entering a cell would have become linked to molecules of calf thymus carrier DNA, rather than plasmid, because of their relative amounts in the precipitate. No results were presented which allow any conclusions to be drawn about these points.

The pBR328/BPV/TK2 vector was constructed because results had shown that the BPV-1/BamHI linearised DNA could recircularise and, in the main, replicate as an episomal form when introduced into mouse C127 fibroblasts. Analysis by shearing or HpaI digestion of DNA extracted from TK^+ Ltap207 transformants containing this vector suggested that the vector DNA had become arranged in a high molecular weight complex which was probably integrated into a host chromosome. Centrifugation of 50μg of total DNA from one cell line (*dam*$^-$ clone 2) through a CsCl/Ethidium bromide gradient (in a similar manner to that described for the C127 pΔBR328/BPV_I/Cl 2 cell line - see Chapter 7.2) failed to resolve the presence of any extrachromosomal supercoiled forms. This again suggests that the vector DNA had integrated into the host genome (results not shown). The use of either *dam*$^+$ or *dam*$^-$ forms of the vector appeared to have no effect on the behaviour of the vector in these TK^+ cell lines although one cell line (*dam*$^-$ clone 3 - see Fig. 3.3A, lane 3) may have had an extrachromosomal rearranged form present. The fact that C. Tyler-Smith (unpublished data) could only recover ampicillin resistant plasmid into *E.coli* at very low frequency suggested that any extrachromosomally replicating forms were either rearranged and/or present at a low copy number. This result agrees with that of Sekiguchi *et al* (1983) who found that the frequency of recovery of a pBR/BPV/TK

vector from transformed cell lines was so low as to suggest that any intact extrachromosomal vector must have been present at less than one copy per cell.

Introduction of the same pBR328/BPV/TK2 vector into C127 cells followed by selection for focus formation, resulted in the deletion of most of the plasmid and TK sequences in nearly all the 10 cell lines examined. HpaI digestion revealed that in most cases the vector had lost between 9 and 6kb of DNA resulting in extrachromosomal forms of between 6 and 9kb in size. Again, a large amount of the vector material was found to be present in a high molecular weight form, which could represent vector sequences either integrated into a host chromosome or present as large extrachromosomal structures. HpaI digestion converted all the BPV-1 containing material to a single major band in each case, although in some cases other fainter bands were detected, which is suggestive of integration, minor rearrangements in extrachromosomal forms or both.

Thus these results suggest that on selection for BPV-1 transformation of C127 cells, the presence of pBR328 and/or TK sequences in the same plasmid were inhibitory to the function of BPV-1 and that only by deleting these sequences could the viral sequences replicate efficiently as extrachromosomal forms in C127 cells. The fact that no intact episomal forms could be detected in the Ltap207 TK^+ transformants suggests

that either the presence of TK or the pBR328 sequences inhibited the replication of the vector, or that there must be selection for the BPV-1 transformation function in order for the extrachromosomal replication to function correctly. Perhaps this is not possible in L cells which are already highly transformed.

The transforming gene of BPV-1 is thought to code for the E2 protein. If selection for transformation is necessary in order to maintain extrachromosomal replication, deletion of the E2 gene should also result in the loss of the ability of BPV-1 to replicate extrachromosomally. Recently Lusky and Botchan (1984) have reported that a BPV-1 molecule in which the E2 transforming gene had been deleted can be maintained in either C127 or LTK$^-$ cells following co-transformation with an unlinked plasmid bearing a selectable marker. These transformants maintained the deleted form of BPV-1 as a extrachromosomally replicating form, even though there is thought to be no selection for these viral sequences and no foci appeared. This result suggests that BPV-1 DNA can replicate extrachromosomally even in the absence of selection for viral transformation. It also suggests that expression of the BPV-1 E 2 protein may be inhibitory to viral replication in transformed lines such as Ltap207. It would therefore seem most likely that, in the case of the vector pBR328/BPV-1/TK, either it was the presence of plasmid or the TK gene or it was the precise arrangement of the plasmid, TK gene

and the viral sequences within the vector that was somehow inhibitory to the extrachromosomal replication of this vector in Ltap207 cells, and that selection for the expression of BPV-1 sequences is not a prerequiste for extrachromosomal replication.

Since the above experiments using the pBR328/BPV/TK2 vector were performed, it is apparent (from the published literature) that the inclusion of the HSV/TK gene in BPV-1 vectors is in someway inhibitory to its extrachromosomal replication in TK^+ transformants. For example the introduction of the HSV/TK gene into the vector pBrdBPV-β_1, DiMaio et al, 1982 (see Introduction) apparently causes it to form a high molecular weight structure which may be integrated into host chromosomes (D. Di Maio, pers. comm.). Similarly, results have been reported by Lusky et al (1983) in which linkage of pML2, HSV/TK and BPV69% fragment in one vector resulted in the formation of a high molecular weight structure with no detectable episomal forms. Even co-transfection of a vector pGP (pBR327/BPV69%/rat growth hormone) with unlinked HSV/TK gene with selection for TK expression resulted in the linkage of the BPV vector into a high molecular weight structure in mouse L cells (Kushner et al, 1982). The pGP vector had been shown to replicate extrachromosomally when selected for focus formation in C127 cells.

The only reported case in which a BPV based vector is found to replicate extracromosomally in the presence

of the HSV/TK gene is that of the E2 deleted vector of Luskey and Botcham (1984) when transformants were selected on the basis of a co-transferred marker. It would, therefore, appear that the only remaining explanation for the observed aggregation of the BPV-1 vectors into a high molecular weight form discussed above is that linkage of the TK and/or plasmid sequences to BPV-1 somehow inhibits its extrachromosomal replication.

In the following Chapter several less complicated vectors are constructed, which contain various bacterial plasmid sequences linked to the complete BPV-1 genome. These vectors were tested for their ability to replicate as intact extrachromosomal forms in C127 cell upon selection for focus formation.

CHAPTER 4

THE INHIBITORY EFFECTS OF PLASMID SEQUENCES UPON BPV-1 VECTORS?

4.1. Introduction

In the previous Chapter it was shown that a proportion of linear BPV-1 DNA replicates as extra-chromosomal monomeric circular forms after introduction into mouse C127 cells. The possibility that some of the high molecular weight viral DNA complex is integrated into a host chromosome was not disputed and shall be investigated thoroughly in Chapter 7. Because of these results, and since many other groups maintain that BPV-1 DNA is present exclusively as extrachromosomal circular forms in transformed cell lines (Law *et al*, 1981, see Chapter 1 for others), the pBR328/BPV/TK2 vector was constructed. This was introduced into two mouse cell lines, C127 and Ltap2O7, and transformants selected either by focus formation or in HAT medium. As seen this vector did not function in the required manner in either cell line. The results suggested that the TK gene and/or plasmid sequences were in some way incompatible with the maintenance of the vector as a nonintegrating, extrachromosomal intact circular form.

As discussed in Chapter 1 DiMaio *et al* (1982) and others suggested that bacterial plasmid sequences are inhibitory to the transformation of C127 cells with plasmid linked BPV-1 and that any rare transformants obtained with these plasmids contain deleted, rearranged extrachromosomal circular forms. This Chapter describes the results of experiments designed to

investigate this inhibitory effect of bacterial plasmid sequences, but in order to do this, simple vectors, consisting of just plasmid (pBR328 or pAT153) sequences linked to BPV-1 DNA, were used to transform C127 cells. In the construction of these vectors the complete BPV-1 genome is used as a BamH1 linear fragment for consistency with the vectors of Sarver et al (1982).

All extrachromosomal circular forms present in cell lines transformed with these initial vectors were found to be deleted forms, so the possibility of a common inhibitory 'poison' sequence was investigated by deleting specific regions from both plasmids. When these 'deletion' vectors also failed to be maintained as intact extrachromosomal circular forms in transformed cell lines an explanation other than the presence of poisonous plasmid sequences was sought to account for the rearrangment of these vectors. On consideration of the results obtained with vectors constructed here, and those reported in the literature, it was concluded that it is the positions of specialized BPV-1 sequences relative to the plasmid sequences and other BPV-1 regions in the vector that is important for vector integrity rather than the presence of specific poison sequences that prevents the original pBR328/pAT153 BPV-1 hybrid vectors from remaining intact in transformants. This hypothesis was tested by manipulating the arrangement of plasmid and BPV-1 DNA sequences in one of the vectors and introducing the constructs into C127

cells. This resulted in the presence of some unrearranged monomeric forms in the DNA of transformed cell lines. Most of these plasmids were introduced by both the calcium phosphate precipitation and microinjection techniques. A detailed account of the reason behind the construction of the various vectors and their behaviour in transformed cell lines is presented in each section.

4.2. Transformation of mouse C127 cells with pBR328/BPV_I and pAT153/BPV_I vectors

The vectors pBR328/BPV_I and pAT153/BPV_I were constructed as in Fig. 2.3 and Section 2.13.5.d. and Fig. 2.5 and Section 2.13.5.k. respectively.

Both pBR328 and pAT153 (Soberon *et al*, 1980; Twigg and Sheratt, 1980) do not contain the so-called 'SV40 poison' sequences, which were found by Lusky and Botchan (1981) to inhibit the replication of SV40-pBR322 hybrid DNA in monkey cells (Rigby, 1982). It was felt that such inhibitory effects could also affect the replication of BPV-1. These vectors were introduced into C127 fibroblasts only by the calcium phosphate precipitate method in the absence of carrier DNA. Separate transformation experiments were set up using both the *dam*$^+$ or *dam*$^-$ forms of the plasmids. The transformation frequencies obtained with these plasmids were of the order of 10-15 foci per μg of BPV-1 per 5.5

x 10^4 cells DNA presented with the precipitate (see Table 2.1). This contrasts sharply with transformation frequencies obtained by others using similar vectors, where it was reported that foci were rarely obtained. In these cases transformation frequencies were often found to be less than one focus per μg of BPV-1 DNA per 5-10 x 10^5 cells (DiMaio et al, 1982; Binetruy et al, 1982; Sarver et al 1982 and Kushner et al, 1982). This discrepancy probably results from variations in the method of precipitate formation used. DiMaio et al (1982), Binetruy et al (1982) and Sarver et al (1982) use a final precipitate consisting of 2.5μg of recombinant and 25μg of carrier DNA per ml precipitated onto approximately 2 x 10^6 cells. Kushner et al (1982) omitted the carrier and their precipitate only contained small amounts of DNA (2.5μg/ml or less). In the present work, carrier DNA was also omitted, but was replaced by a similar quantity of the recombinant DNA so that the final precipitate contained carrier free vector DNA at a concentration of 20μg/ml. In each transformation experiment 30μg of the recombinant DNA was precipitated onto 1 x 10^6 cells (see Section 2.1.2.E).

The fact that here a large excess of vector DNA was used in these experiments increased the chances of obtaining transformants, but decreases the measured efficiency per μg of DNA because of the large excess of the vector present. As a result of these differences,

the transformation frequencies presented here can only be compared internally and cannot be directly compared with those presented by other groups.

No significant difference in the transformation frequency was seen between that obtained with intact pBR328/BPV$_I$ above and that seen in the previous Chapter (see Table 2.1) using BPV-1 cleaved away from the plasmid sequences (10-15 foci/μg of BPV-1 DNA/5 x 10^4 cells in both cases). Again this is contrary to the reports of many other groups where it is accepted that linkage of BPV-1 to bacterial plasmid sequences significantly decreases the numbers of transformants obtained, by usually 100-300 fold. However, it must be noted that the transformation system used in the present work was not set up to obtain maximal transformation efficiencies per μg for comparison between different vectors, but just to obtain sufficient foci to establish transformed cell lines.

4.3. Analysis of Cellular DNA in pBR328/BPV$_I$ and pAT153/BPV$_I$ C127 Transformants

The DNA from four independently derived pBR328/BPV$_I$ C127 transformants was analysed by mild shearing, gel electrophoresis and blot hybridisation with a homologous probe. The analysis revealed that all the vector sequences were present in a high molecular weight

complex, similar to that seen in the Ltap207 TK^+ transformants in the last chapter except at a much greater copy number (~20-50 results not shown). No extrachromosomal forms represented by fast migrating bands could be detected in these DNA samples. HpaI digestion (1 cleavage site within the BPV-1 sequences, Fig. 4.1.a) revealed the presence of a single major band in each of these DNAs (lanes 1 to 4), which comigrated with the 12.85kb linear form of the input vector (i.e. the upper most band in lane X). At least two other minor bands are visible in each of these DNAs, and a longer exposure of the autoradiograph reveals the presence of many other bands (data not shown). These minor bands could be derived from integrated vector sequences, generated by HpaI digestion of a vector/host DNA junction, or they could be derived from digestion of rearranged/deleted forms which themselves could be integrated or form part of a very large extrachromosomal structure. Thus it is possible that vector sequences are integrated, organised into a large extrachromosomal structure or both. The fact that the major HpaI band is identical to the size of the input DNA suggests that in the cell the vector units are organised as concatemeric, tandem head to tail structures. The use of pAT153 in a BPV-1 vector controls for any inhibitory effect that the chloramphenicol gene in pBR328 might have on the BPV-1 vector.

A similar analysis of C127 pAT153/BPV_I (<u>dam</u>$^+$

FIGURE 4.1. pBR328/BPV$_I$, pΔBR328/BPV$_I$, and pΔIR328/BPV$_{II}$ DNAs in C127 focus selected cell lines.

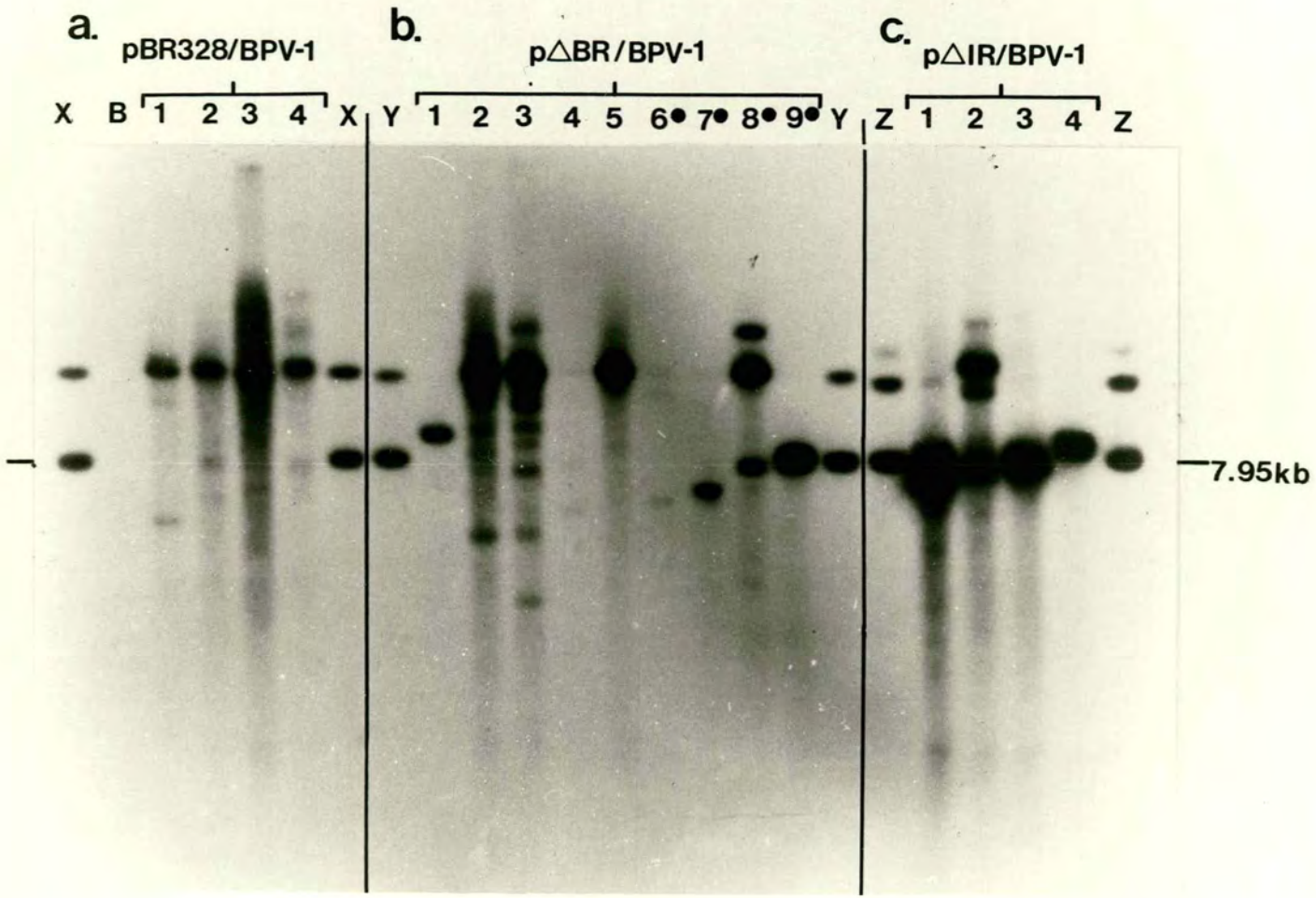
Cellular DNA (5μg) was digested with a fivefold excess of HpaI. Samples were electrophoresed through a 0.7% agarose gel, transferred to nitrocellulose and hybridised to a nick translated probe, pBR328/BPV-1/TK$_2$.

Samples:- B; Ltap207 HpaI digested non transformed total cellular DNA. X,Y,& Z; 10 copy equivalent of BamHI linearised BPV-1, and HpaI linearised plasmids pBR328/BPV$_I$ (X), pΔBR328/BPV$_I$ (Y), or pΔIR328/BPV$_{II}$ in 5μg of HpaI digested Ltap207 DNA.
a. 1; DNA extracted from C127 pBR328/BPV$_I$ dam$^+$ Clone 1. 2; Clone 3. 3; Clone 4. 4; Clone 5.
b. 1; DNA extracted from C127 pΔBR328/BPV$_I$ dam$^+$ Clone 3. 2; Clone 4. 3; Clone 5. 4; DNA extracted from pΔBR328/BPV$_I$ dam$^-$ Clone 1. 5; Clone 6. 6; dam$^+$ Ij 32A. 7; Ij 32B. 8; Ij 36A. 9; Ij 36B.
c. 1; DNA extracted from C127 pΔIR328/BPV$_{II}$ dam$^+$ clone 1. 2; clone 2. 3; clone 4. 4; clone 5.

Ij indicates cell lines derived by microinjection, marked by a spot • in figure. All other cell lines were derived by calcium phosphate precipitation.

dam$^+$ indicates input plasmid was grown in E.coli HB101 dam$^+$.
dam$^-$ indicates input plasmid was grown in E.coli CSH26 dam$^-$.

Hpa-1 DIGESTION OF C127 FOCUS SELECTED CLONES.



or dam$^-$) transformants also revealed that in some of the sheared DNA samples only high molecular weight smears were present. In the other DNAs episomal forms were also found (data not shown), but these were all of a size smaller than the input vector DNA. Differential probing of sheared samples (i.e. in a similar analysis to that in Fig. 3.3) revealed that bacterial plasmid sequences (pAT153) had been deleted from most of the episomal circular forms and that in some cases the BPV31%NT HindIII/BamHI region was also effected (data not shown). The results of HpaI digestion of those DNAs which contained the episomal forms in addition to the high molecular weight form, is shown in Fig. 4.2.a. Of the DNAs from the seven cell lines (lane 6 and 7 contain subclone and clone respectively) those in lanes 1, 2 and 4-7 show the presence of a band of varying intensity which migrates at ~11.3kb, which corresponds to the position of linearised pAT153/BPV_I input DNA. Along with this, bands, which in some cases represent major forms are seen between 6 and 9kb in lanes 3, 4, 6, 7 and 8, (i.e. lane 3 has one at ~6 and another at 9kb). These DNA samples correspond to those that were seen to contain circular forms after mild shearing. Thus, the monomeric episomal forms are about the size of BPV-1 (6- 9kb), but whether or not the high molecular weight smear seen in all these lines is extrachromosomal or integrated is again not known. The other minor bands seen in these DNAs are again indicative of rearranged, deleted episomal or integrated forms.

FIGURE 4.2. pAT153/BPV$_I$ (A, left panel), and pΔAT153/BPV$_I$ & II (B, right panel) DNAs in C127 focus selected cell lines.

Cellular DNA (5μg) was digested with a fivefold excess of HpaI. Samples were electrophoresed through 0.6% agarose gels, transferred to nitrocellulose and hybridised to a nick translated probe, pBR328/BPV-1/TK$_2$. All these cell lines were derived by calcium phosphate precipitation.

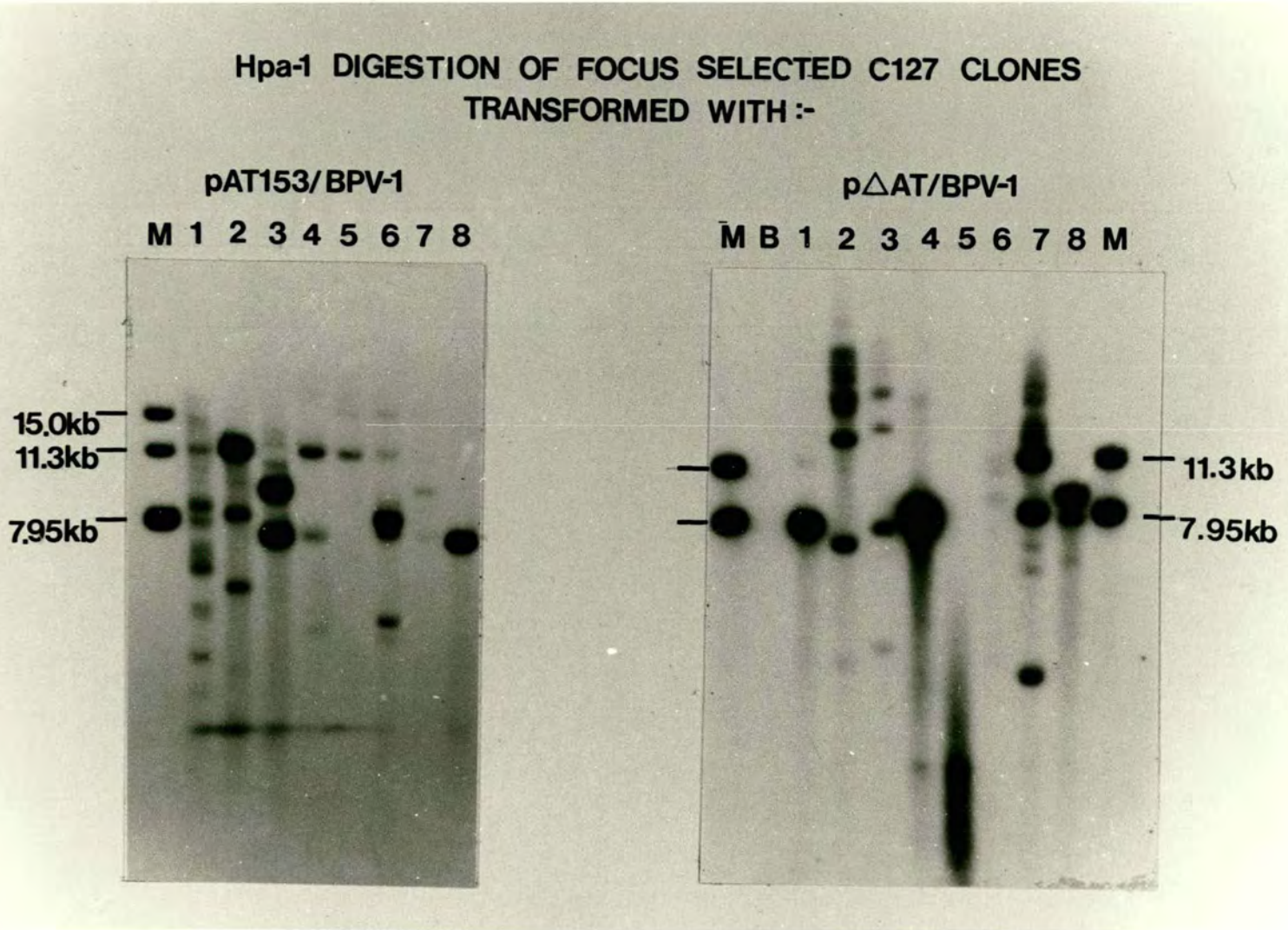
Samples:-

A.(left panel) : M; 10 copy equivalent of BamHI linearised BPV-1 (7.95Kb), and HpaI linearised plasmids pAT153/BPV$_I$ (11.3Kb.) and pΔBR328/BPV$_I$/TK$_2$ (15.0Kb), in 5μg of HpaI digested Ltap207 DNA. 1; DNA extracted from C127 pAT153/BPV$_I$ dam$^+$ Clone 1 pl 1 rc 2; Clone 2 pl 1 pool. 3; Clone 4 rc. 4; Clone 6 pl 1 pool. 5; dam$^-$ Clone 7 rc. 6; Clone 8 pool. 7; Clone 8 rc. 8; Clone 2 pl 1 rc.

dam$^+$ indicates input plasmid was grown in E.coli HB101 dam$^+$.

dam$^-$ indicates input plasmid was grown in E.coli CSH26 dam$^-$.

B.(right panel) : M; 10 copy equivalent of BamHI linearised BPV-1 (7.95Kb), and HpaI linearised plasmids pΔAT153/BPV$_{II}$ (11.3Kb) in 5μg of HpaI digested Ltap207 DNA. B; Ltap207 HpaI digested non transformed total cellular DNA. 1; DNA extracted from C127 pΔAT153/BPV$_I$ Clone 1. 2; Clone 2. 3; Clone 5. 4; Clone 6. 5; DNA extracted from C127 pΔAT153/BPV$_{II}$ Clone 1. 6; Clone 4. 7; Clone 5. 8; Clone 6.


Hpa-1 DIGESTION OF FOCUS SELECTED C127 CLONES
TRANSFORMED WITH :-
pAT153/BPV-1
M 1 2 3 4 5 6 7 8
15.0kb
11.3kb
7.95kb
pΔAT/BPV-1
M B 1 2 3 4 5 6 7 8 M
11.3kb
7.95kb

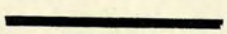
4.4. Transformation of mouse C127 cells with $p\Delta BR328/BPV_I$, $p\Delta IR328/BPV_{II}$ $p\Delta AT153/BPV_I$ and $p\Delta AT/BPV_{II}$

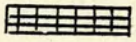
The above results suggest that the absence of the HSV/TK gene from the $pBR328/BPV_I$ construct still did not allow the vector to replicate as an intact episomal circular form in C127 focus selected transformants. Because pBR328 is larger than, and contains other sequences not present in, pBR322 or derivatives of it, including pAT153 used here (Fig. 4.3), the use of the $pAT153/BPV_I$ vector controls for any effect that the 'extra' sequences of pBR328 might have on the replication of the vector in transformants. However, the behaviour of the pAT153 based vector in C127 transformants was very similar to that observed in the C127 cells transformed with pBR328/BPV/TK2 and analysed in Chapter 3. Any small extrachromosomal forms were found to have lost most or all of the plasmid sequences, although HpaI digestion revealed the presence of a larger number of minor bands in the $pAT153/BPV_I$ transformants. Therefore, it would seem that removal of the HSV-TK gene, or the absence of the pBR328 chloramphenicol gene by the use of pAT153, did not overcome the deleterious effect that linkage of bacterial plasmid sequences to BPV-1 may have been exerting upon vector replication.

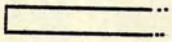
FIGURE 4.3. Physical map of the various pBR322 derivatives indicating those sequences which have been conserved.

: pBR322 sequences remaining in each plasmid derivative.


: deleted pBR322 sequences.

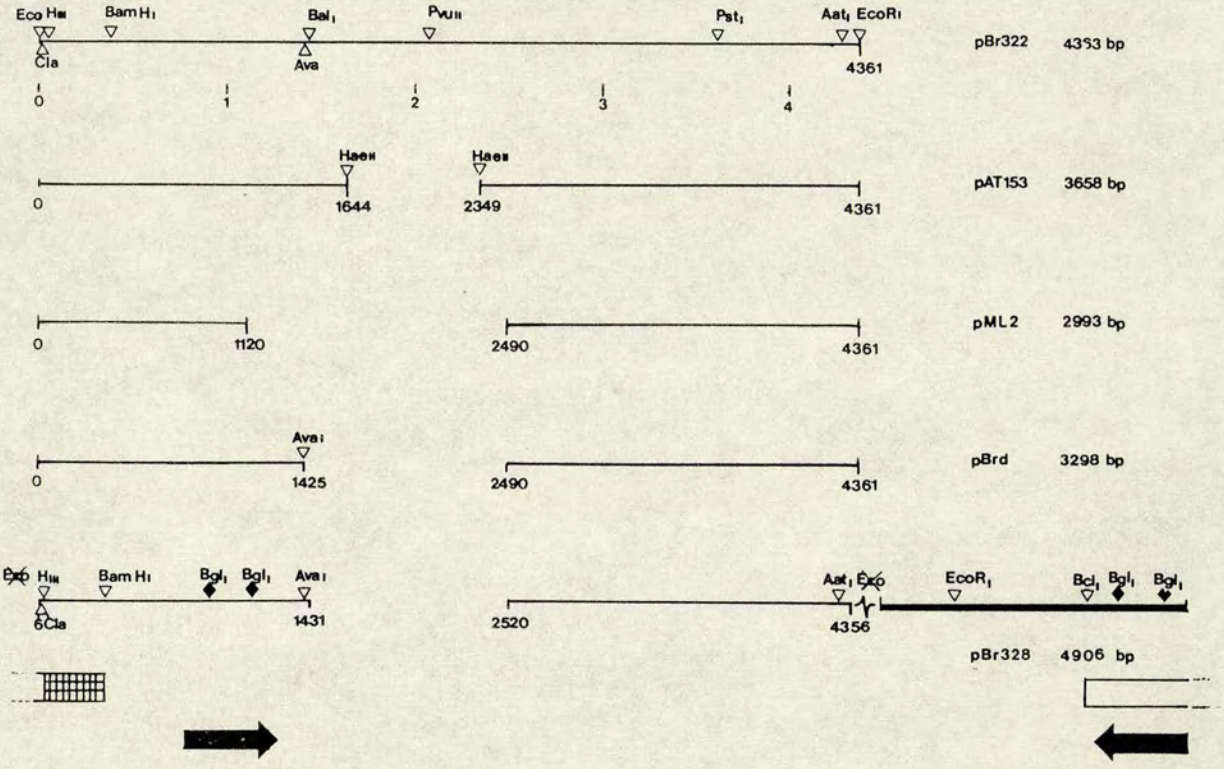

: insert containing the the chloramphenicol gene in pBR328.


: indicates position of the deleted 344bp HindIII/BamHI fragment in each plasmid.


: indicates position of the additional plasmid sequences deleted in pΔIR328/BPV_{II}.


: indicates position of inverted repeat in pBR328.

Restriction endonuclease sites in, and sizes of the plasmids are as indicated. Compiled with reference to Bolivar et al (1976, 1977a), Bolivar (1978), Bolivar and Backman (1979), DiMaio et al (1982), Lusky and Botchan (1981), Mellon et al (1981), Sarver et al (1982), Soberon et al (1980) and Twigg and Sherratt (1980).


Eco HIII
BamH1
Bal1
PvuII
Pst1
Aat1 EcoR1
Cla
Ava
4361
0
1
2
3
4
pBr322
4353 bp
HaeIII
Hae3
1644
2349
pAT153
3658 bp
1120
2490
pML2
2993 bp
Ava1
1425
pBrd
3298 bp
Exo
HIII
Bgl1
6Cla
2520
4356
EcoR1
Bcl1
pBr328
4906 bp

As discussed in the Introduction, Sarver et al (1982) found that a vector, which consists of BPV-1 DNA inserted into the BamHI site of pML2d (Fig. 4.3) and which is similar to pAT153/BPV_I used here, would transform C127 cells with the same efficiency as linear BPV-1 DNA. This vector (pML2d BPV-1 [see Fig. 2.10]) replicates as a stable, multicopy, circular unrearranged plasmid in transformed mouse C127 cells. In contrast they found a similar vector based on the 'parental' pBR322 (pBR322 BPV-1) , gave rise to foci at a very low frequency. The DNA from one pBR322 BPV-1 transformant contained deleted episomal forms of the input plasmid. The deletions were of plasmid sequences; one 2.5kb deletion of this cell line being mapped as a 2kb deletion from the large BamHI-PvuII fragment and a 0.5kb deletion from the small BamHI/PvuII fragment of pBR322. This 2kb deletion of plasmid sequences overlaps with the pBR322 sequences deleted from pML2, which led to the claim that these so called 'SV40 poison sequences' were also inhibitory to BPV-1 replication. However, Binetruy et al (1982) also constructed vectors containing the complete BPV-1 genome cloned as a BamHI (pMB4) or a HindIII (pMH4) fragment inserted into pML2. Contrary to the results of Sarver et al (1982), it was found that both vectors transformed rat FR3T3 or mouse C127 cells at a "very low frequency" using the calcium phosphate precipitation technique. In fact none were obtained. Transformants were eventually obtained by the protoplast fusion technique (Schaffner et al, 1980;

Rassoulzadegan et al, 1982), when some were seen, following digestion with restriction enzymes, to contain deleted forms of the input vector and others contained intact vector. None of the vector sequence was present as monomeric circular forms, but as heterogeneous oligomeric forms which were said to be unintegrated since no junction fragments could be detected.

These results suggest that removal of the 'SV40 poison' sequences by use of pML2 did not remove the inhibitory effect that these sequences seem to have on transformation or the replication of the vector as an intact monomeric circular form. They also suggest that there must be some major difference between the vectors used by these two groups. Close examination of the construction of pML2d BPV-1 by Sarver et al (1982) revealed that the small 344bp HindIII/BamHI fragment of pML2 had been deleted (d; for reasons of vector consistency) but had been retained in both vectors pMH_4 and pMB_2 constructed by Binetruy et al (1982). Thus, it was possible that the presence of this small 344bp fragment was responsible for the malfunction of pBR322 BPV-1 (Sarver et al, 1982) pMH_4, pMB_2 (Binetry et al, 1982) and those vectors constructed here in this thesis ($pBR328/BPV/TK_2$, $pBR328/BPV_I$ and $pAT153/BPV_I$). To test this hypothesis a set of new vectors were constructed from which this 344bp fragment was deleted (see Figs. 2.3, 2.4, 2.5 and Section 2.13.5 for details). These were $p\Delta BR328/BPV_I$ (Fig.

2.4) and pΔAT153/BPV$_{I\ \&\ II}$ (Fig. 2.5) where Δ refers to the fact that the HindIII/BamHI 344bp fragment has been deleted. A vector pΔIR328/BPV$_{II}$ was also constructed (Fig. 2.4) from which, in addition to the 344bp fragment; one copy of the 482bp inverted repeat of pBR328 (Prentki et al, 1981 - see Fig. 4.3) was also removed. It was felt that the presence of an inverted repeat of this size in the vector could cause rearrangements.

These four vectors were then introduced into C127 by the calcium phosphate precipitation method, and foci selected, pΔBR328/BPV$_I$ was also introduced by microinjection. The results obtained on expansion of foci, extraction of total cellular DNA and analysis by blot hybridisation are presented below.

4.5 Analysis of Cellular DNA in pΔBR328/BPV$_I$, pΔIR328/BPV$_I$, pΔAT153/BPV$_I$ and pΔAT153/BPV$_{II}$ transformants

Analysis of total cellular DNA from several independently derived cell lines transformed with any of the above vectors gave very similar results. Mild shearing of the DNA, followed by probing with a labelled homologous sequence (i.e. BPV-1) showed that in some cell lines transformed with pΔBR328/BPV$_I$ by either method the vector sequences were present only as a high molecular

weight complex forming a smear of hybridisation. In other cell lines, extrachromosomal circular forms could be detected in addition to this high molecular weight complex, but they migrated faster than the input vector DNA (data not shown).

HpaI digestion of these DNAs (only one cleavage site in pΔBR328/BPV$_I$ Fig. 4.1.b.) revealed that, in general, in those cell lines containing only the high molecular weight complex, the hybridising sequences were converted to a major linear band which comigrated with the linear form of the input vector (i.e. 12.5lkb, upper most band in lanes Y, Fig. 4.2.b.). It is also noticeable that, in these cell lines, many other minor bands can be seen which must represent either rearranged and/or deleted forms which are joined together in a large structure which might be integrated. When 50μg of cellular DNA from one of these DNAs (Clone 4, lane 3) was fractionated on a CsCl ethidium bromide gradient only one high molecular weight form of vector DNA could be detected, and only in the open circular chromosomal DNA fractions. No vector sequences could be detected in other fractions which would have contained circular forms. This suggests that the vector sequences are either integrated into the host genome or present as very large extra-chromosomal forms in this and, perhaps, other cell lines (data not shown).

HpaI digestion of DNA from the remaining pΔBR328/BPV$_I$ transformants, which contain episomal circles in

addition to the high molecular weight complex, converted most of the hybridising DNA to a major band of between ~7 and 9kb (Fig. 4.2.b, lanes 1, 6, 7 and 9, lower band in Y is BPV-1 linear). Some of these DNAs contained other faint bands while in others no such bands were detectable. Interestingly, in Fig. 4.1.b. lane 8 (derived from a microinjected cell) the major HpaI band comigrates with linearized input vector DNA. When analysed by shearing this DNA was found to contain a major episomal circular form which comigrates with the supercoiled form of the input vector (data not shown). Thus, this cell line may contain some extrachromosomally, replicating intact pΔBR328/BPV$_I$ vector, but it is also possible that the apparently intact vector in fact represents a dimeric rearranged form. This shall be investigated by plasmid recovery experiments (see Chapter 6.2.D).

Similar results have been found on analysis of total DNA extracted from the pΔIR328/BPV$_{II}$ transformants. Mild shearing showed the presence of both extrachromosomal deleted circular forms which migrated faster than the supercoiled form of the input DNA, together with high molecular weight forms (Fig.4.4). HpaI digestion of 4 of these DNAs (Fig.4.1.C) resulted in their conversion to a linear form, which migrated at approximately the same position as the 7.95 kb BPV-1 linear fragment. Once again this suggests the presence of a deleted form of the vector in each cell line and that most of the bacterial plasmid sequences have probably been lost. As before, minor bands

FIGURE 4.4. $p\Delta IR328/BPV_{II}$ DNA in C127 focus selected cell lines.

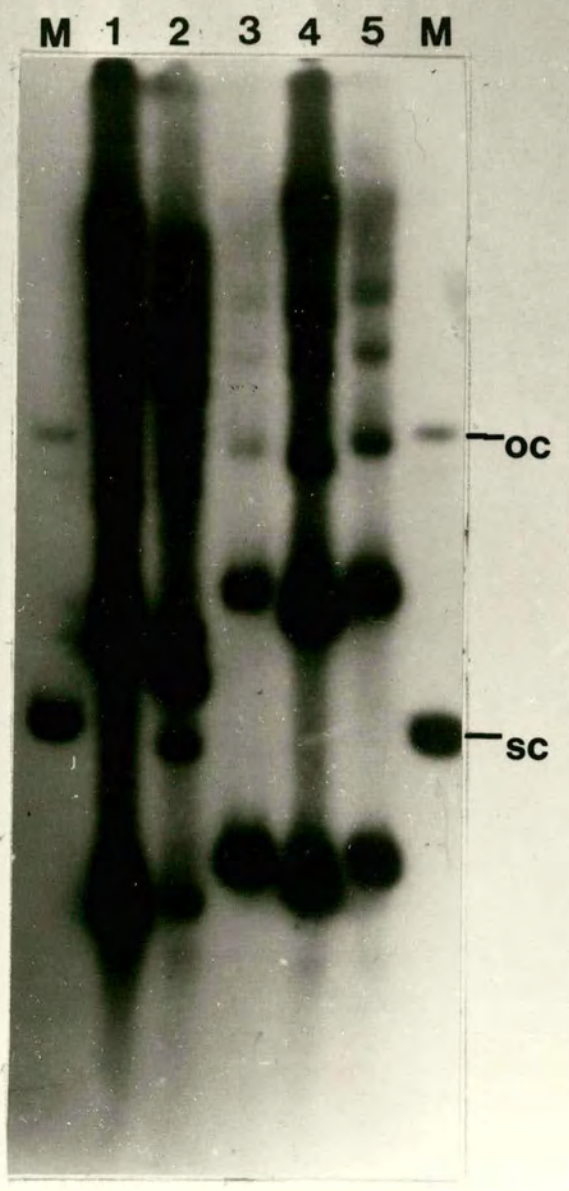
Cellular DNA (5µg) was sheared by passage through a 25 gauge syringe. Samples were electrophoresed through a 0.6% agarose gel, transferred to nitrocellulose and hybridised to a nick translated probe, pBR328/BPV-1/TK_2. All these cell lines were derived by calcium phosphate precipitation.

Samples:-

M; 10 copy equivalent of undigested plasmid $p\Delta IR328/BPV_{II}$ (12.5Kb.) in 5µg of HpaI digested Ltap207 DNA.

1; DNA extracted from C127 $p\Delta IR328/BPV_{II}$ Clone 1.
2; Clone 2. 3; Clone 4. 4; Clone 5. 5; Clone 6.

C127pΔIR328/BPV II FOCUS SELECTED.



could be detected in some of the DNAs, for example, clone 2 (Fig. 4.1.C. lane 2) contained two forms which comigrated at a position similar to that of the linear form of the $p\Delta IR/BPV_{II}$ vector (11.96kb - top (heavy) band, lane Z). Again these may represent dimeric circular rearranged forms, although one (the smaller) of these could be basically intact $p\Delta IR328/BPV_{II}$ but with a small deletion, since it migrates close to the position of the supercoiled form of the input vector in Fig. 4.4., lane 2.

Analysis of total cellular DNA extracted from $p\Delta AT153$ $BPV_{I\&II}$ transformants by mild shearing and blot hybridisation, showed the presence of deleted supercoiled circular forms in the 6 $p\Delta AT/BPV_{I}$ (orientation I OF BPV) and 5 $p\Delta AT/BPV_{II}$ (orientation II of BPV) DNAs (Fig. 4.5). All cell lines also contained higher molecular weight forms. HpaI (only 1 cleavage site) digestion (Fig. 4.2.B.) of DNA of seven of these cell lines confirmed that in each case the major form of vector sequences was a molecule of size similar to linear BPV-1 DNA (i.e. 7.9kb) but, in any case, smaller than the 11.3kb input vector DNA . Other faint bands (of size greater than 11.3kb) were detectable in all cell lines, but, many of these could have resulted from partial digestion of larger oligomeric forms (lanes 2, 3, 6 and 7). The cell line in lane 7 (C127 $p\Delta AT/BPV_{II}$ clone 7) also contained a major band at 11.3kb which could represent a dimer of a rearranged form or intact $p\Delta AT/BPV_{II}$ derived from a band seen to comigrate with the intact supercoiled form in Fig. 4.5, lane 11.

FIGURE 4.5. $p\Delta AT153/BPV_{I\ \&\ II}$ DNA in C127 focus selected cell lines.

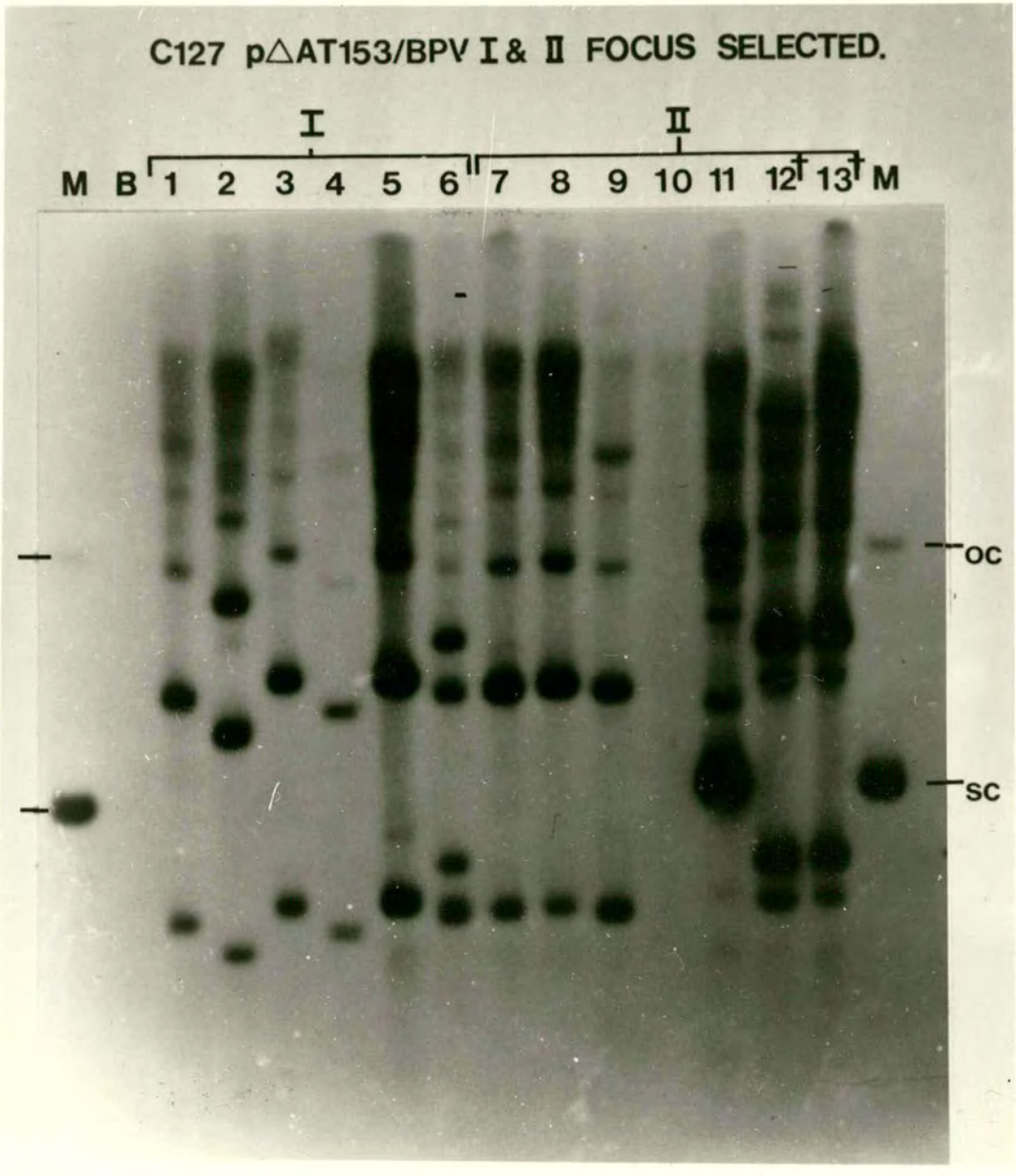
Cellular DNA (5 μg) was sheared by passage through a 25 gauge syringe. Samples were electrophoresed through a 0.6% agarose gel, transferred to nitrocellulose and hybridised to a nick translated probe, $pBR328/BPV\text{-}1/TK_2$.
All these cell lines were derived by calcium phosphate precipitation.

Samples:-

M; 10 copy equivalent of undigested $p\Delta AT153/BPV_I$ (11.3Kb.) input plasmid in 5μg of sheared Ltap207 DNA. B; 5μg of sheared Ltap207 DNA.

1; DNA extracted from C127 $p\Delta AT153/BPV_I$ Clone 1. 2; Clone 2. 3; Clone 4. 4; Clone 5. 5; Clone 6. 6; Clone 3. 7; DNA extracted from C127 $p\Delta AT153/BPV_{II}$ Clone 1. 8; Clone 2. 9; Clone 3. 10; Clone 4. 11; Clone 5. 12*; Clone 6. 13*; Clone 6.

†: indicates that DNA samples were derived from the same cell line.


C127 pΔAT153/BPV I & II FOCUS SELECTED.
I
II
M B 1 2 3 4 5 6 7 8 9 10 11 12 13 M
oc
sc

The results obtained with all the Δ deleted vectors suggest that the deletion of this fragment from the plasmid DNAs does not lift the inhibitory effect that the plasmid sequences seem to have on the replication of intact circular vector. The failure of the pΔBR328/BPV_I vector to remain intact in transformed C127 cells, might have been explained by the presence of the chloramphenicol resistance gene or the inverted repeat in the plasmid sequences. However, both these possibilities are removed by the fact that both pΔAT153/$BPV_{I\&II}$ (which contains no chloramphenicol resistance gene and no inverted repeat) and pΔIR328/BPV_{II} (which contains no inverted repeat) also result in deletions. On comparison of the plasmid sequences present in all these vectors (Fig. 4.3) it can be seen that the 'SV40 poison sequence' deletion in pML2 is much larger than that in pAT153, in fact pAT153 retains 665bp more of the 'parental' pBR322 vector sequences than does pML2. The deletion in pBR328 covers most of the sequences missing from pML2, but still retains 305bp of the pBR322 sequences not present in pML2 and, in addition, contains the chloramphenicol resistance gene and the inverted repeat sequences. Thus, it is conceivable that in the vectors constructed here the smaller deletions in pAT153 and pBR328 although sufficient to allow replication of SV40-pAT153 or SV40-pBR328 hybrid molecules in monkey cells (Rigby, 1982), are not sufficient to allow replication of BPV-1-pAT153 or BPV-1-pBR328 hybrid vectors in mouse C127 cells. Therefore, the possibility still remains that the chloramphenicol

marker and/or the inverted repeat might also interfere with the intact replication of BPV-1-pBR328 hybrid vectors.

To test this, the vector pML2d/BPV-1, constructed by Sarver et al (1982) should be introduced into these C127 cells to see if the same result is obtained. The results of analysis of DNAs from C127 cells transformed with pML2d BPV-1 are presented in Chapter 5.

4.6. Transformation of Mouse C127 cells with pΔAT153/BPV$_I$/EN-DUP B, C and D

On consideration of all the vectors constructed during the course of this work, and those in the published literature (see Chapter 1 for discussion), it seemed possible that the vectors were not replicating as intact monomeric episomes for some reason other than the presence of inhibitory plasmid sequences. DiMaio et al (1982) showed that a vector pBRd BPV-β_1 would replicate as a intact monomeric circular form in C127 cells. This vector (see Fig. 2.7) contains the BPV69%T fragment linked to pBRd, which is similar to pML2d (see Fig.4.3.), and a 7.6kb HindIII fragment of human DNA containing the β-globin gene. On the face of it this is surprising since neither Sarver et al (1982) or DiMaio et al (1982) could obtain foci using the BPV69%T inserted in pML2 or pBRd alone, even though the vector pML2d BPV-1 containing the complete BPV-1 genome could transform C127 cells with the same efficiency

as isolated BPV-1 linear (Sarver et al, 1982). Contrary to these results Campo and Spandidos (1983) have found that a vector pBV1-D1 containing the BPV 69%T fragment cloned in pAT153 transforms NIH3T3 cells. Transformation efficiencies were of the order of 19 foci/μg of BPV-1 DNA/5.7 x 10^5 cells when 10μg of recombinant were applied to 5.7 x 10^6 cells. However, although the pBV1-D1 vector was found to be maintained as an extrachromosomal form, analyses of restriction digests revealed that rearrangements had occurred. Rare transformants were obtained with the intact pBRd-BPV69%T (pBPV-HII, DiMaio et al, 1982) vector and were found to contain rearranged episomal forms which had acquired additional DNA sequences. Similar events have been reported by Mitrani-Rosenbaum et al (1983). These observations suggested that the 31% non-transforming region of BPV-1 (BPV31%NT) must be playing some role in the transformation and replication of these vectors and that this role could be replaced by the acquisition of some unknown DNA sequence. Furthermore, DiMaio et al (1982) found that inclusion of a specific DNA sequence, human β-globin 7.6kb fragment, stimulated the transformation efficiency and allowed the vector to replicate in an unrearranged form. Thus it would seem that the human β-globin gene and the BPV31%NT fragment can provide the basic vectors with the same 'function'; one which allows the efficient transformation of, and their replication in, C127 cells. Sarver et al (1982) present a similar argument and have identified three additional eukaryotic sequences which can provide the same 'function' as the β-globin fragment

when substituted for the BPV31%NT fragment. These are the rat preproinsulin gene (5.3kb), the human growth hormone gene (2.6kb) and an intergenic rat DNA fragment (7.3kb). In addition Kushner et al (1982) report a similar effect of the rat growth hormone gene (5.8kb).

On consideration of the effect of these various eukaryotic fragments on the function of a BPV69%T containing vector, it seemed possible that they might all contain a sequence with enhancer properties which might potentiate transcription from the BPV-1 regulatory sequences thought to reside close to the HindIII site (Chen et al, 1982, see Fig. 4.6). This region has also been found to contain a prominent DNAase-1 hypersensitive site (Rosl et al, 1983) and a plasmid maintenance sequence or origin of replication (Lusky and Botchan, 1984; Waldeck et al, 1984). Moreover, observations made on the predicted amino acid sequence of the E1 protein of BPV-1 (putative E1 protein reading frame) shows that this E1 'protein' has considerable homology (extending over 200 amino acids) to the functional regions of polyoma virus large T antigen (Clertant and Sief, 1984). The large T antigen of polyoma virus is required for the initiation of polyoma viral DNA replication (Acheson, N. 1980). Thus, the role of the E1 'protein' of BPV-1 might be similar to that of the large T antigen, and indeed it has been shown to be required for replication of BPV-1 in the absence of transformation (Lusky and Botchan, 1984).

FIGURE 4.6. Physical map of the BamHI cleaved BPV-1 genome, showing the relative positions of various functionally defined regions.

PMS 1 & PMS 2 : plasmid maintenance sequences, mapped by Lusky and Botchan (1984) and Waldeck et al (1984).

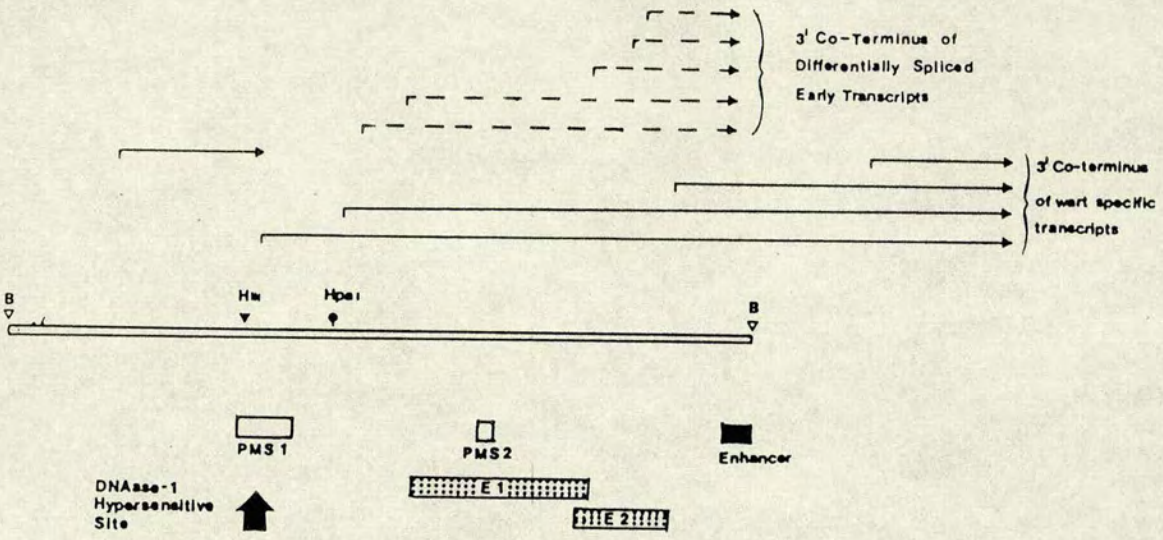
DNAase-1 hypersensitive site indicated by large heavy arrow, mapped by Rosl et al (1983).

Enhancer sequence indicated by dark box was mapped by Campo et al (1983), Lusky et al (1983) and Weiher and Botchan (1984). It resides on a 60bp Sau3A/BamHI fragment.

E 1 : represents the open reading frame thought to encode a protein required for replication (Engel et al, 1983; Clerant and Sief, 1984).

E 2 : represents the open reading frame thought to encode the transformation protein (Engel et al, 1983; Nakabayashi et al 1984; Lusky and Botchan, 1984).

The complete BPV-1 genome has been sequenced by Chen et al (1982.)


3' Co-Terminus of
Differentially Spliced
Early Transcripts
3' Co-terminus
of wart specific
transcripts
B
Hin
Hpa I
B
PMS 1
PMS2
Enhancer
DNAase-1
Hypersensitive
Site
E 1
E 2

It would therefore seem that the overall arrangement of the functional regions within the BPV-1 genome is similar to that in polyoma and SV40 viruses. One important distinguishing feature is that in polyoma and SV40 the viral enhancer sequences are adjacent to the transcriptional regulatory sequence (i.e. the promoter) and replication origin (Moreau et al, 1981; Benoist et al, 1981; Gruss et al, 1981; DeVillier and Schaffner, 1981 and Tyndall et al, 1981) but the enhancer in BPV-1 DNA is some distance from the promoter, lying 5.4kb away in the 3' direction and ~2.5kb away in the 5', in the circular genome. This means that when BPV-1 is cloned into any plasmid as either a BamHI or a HindIII insert, the distance between the enhancer and its regulatory region is automatically increased. Increasing the distance between the SV40 enhancer and its promoter or the conalbumin promoter element has been shown to decrease the transcriptional activity from those promoters (Wasylyk et al, 1984). Wasylyk et al (1983) had previously found that the SV40 enhancer could activate transcription from procaryotic sequences, which behave as a 'substitute' promoters, one of which is present at nucleotide 610 in pBR322. It had also been shown that promoter elements immediately proximal to enhancers are stimulated in preference to more distal promoters (Moreau et al, 1981; Wasylyk et al, 1983).

Taking the above evidence into consideration it is possible that the enhancement of the BPV promoters could be affected in a similar manner. Indeed, Lusky et al (1982,

1983) have shown that on replacement of the SV40 enhancer with the BPV-1 enhancer, transciption of the SV40 early region starts from the same site. Thus, separation of the BPV-1 enhancer from its regulatory sequences at the HindIII site, by insertion into a plasmid (3-5kb), could result in a decrease in transcriptional activity by 99.5% (as reported for SV40 Wasylyk et al, 1984). The 'substitute' pBR322 promoter is present in pBR328, pAT153, pML2d, pBRd and all the deletion derivatives of pBR328 and pAT153 used here in making BPV-1 constructs. Thus it is conceivable that, in all the vectors constructed here, transcription would be activated from this "substitute" plasmid promoter, with the consequence that stimulation at the more distal BPV-1 promoter would be decreased (see Fig. 2.6 "i" is the substitute promoter). Similar difficulties might be encountered if activation of the viral promoter was attempted in the opposite direction i.e. across the 5.5kb (3' to 5') distance between the BPV-1 enhancer and the BPV-1 promoter.

It is therefore possible that the vectors utilised here and elsewhere did not replicate as unit sized circles because of the arrangement of the viral enhancer, the bacterial plasmid sequences and the viral regulatory region. There would therefore be strong selection for forms which spontaneously deleted the plasmid sequences, restoring more closely the natural relationship between BPV-1 enhancer and promoter elements. The vectors such as that of DiMaio et al (1982) which contain the human β-globin gene, may

in fact replicate as intact monomeric forms due to the stimulation of transcription from the BPV-1 promoter by an enhancer type element (or read through transcription) present in the eukaryotic fragment which is placed adjacent to the viral promoter (see Fig.2.7, of pBrd/BPV-β_1).

In order to test this hypothesis, a series of vectors were constructed (Fig. 2.6) which contain a duplication of the BPV-1 enhancer (60 bp BamHI/Sau3A fragment) upstream (5') of the BPV-1 regulatory region in either orientation. pΔAT/BPV$_I$/EN-DUP/C, has the enhancer duplicated in the same orientation as in BPV-1 and pΔAT/BPV$_I$/EN-DUP/D has the enhancers duplicated in the reverse orientation. As a control for the effects of duplication of the BPV-1 enhancer, pΔAT/BPV$_I$/EN-DUP/B was constructed with a duplication of the enhancer in tandem with, but in an inverted orientation relative to the original enhancer. In theory the vectors C and D should, when introduced into C127 cells, overcome any inhibitory effect of increased distance of the BPV-1 enhancer from the promoter, or of the presence of the bacterial plasmid substitute promoter. Transformants resulting from the introduction of pΔAT153/BPV$_I$/EN-DUP/C or D would be expected to contain unrearranged monomeric supercoiled forms of these plasmids. In contrast pΔAT153/BPV$_I$/EN-DUP/B would be expected to give similar results to those observed with pΔAT153/BPV$_{I\&II}$ above. All three vectors were introduced into C127 cells by the calcium phosphate precipitation method, but only C & D were

introduced by microinjection. The state of DNA in transformants is analysed below.

4.7 Analysis of Cellular DNA in pΔAT153/BPV$_I$/EN-DUP B, C or D transformants

Analysis of sheared DNA samples extracted from pEN-DUP-B, pEN-DUP-C and pEN-DUP-D transformants resulting from calcium phosphate precipitation is shown in Fig. 4.7. As can be seen most of these DNAs contain deleted forms of the input vector (11.4kb supercoil and open circular forms shown in lane M) along with the presence of other higher molecular weight forms which may or may not be integrated. These deleted forms are probably 6-9kb in size, as has been seen with many of the other vectors upon digestion with a 1 cut restriction enzyme such as HpaI which cuts only once in the BPV1 genome. In addition to these deleted forms there are bands which comigrate with both the supercoiled and open circular forms of the input vectors. These are seen in many tracks with all three vectors (lanes 3 and 4, with pEN-DUP-D, lanes 6 and 7 with pEN-DUP-C and lanes 12-14 with pEN-DUP-B). In previous transformations with other vectors only 3 cell lines were ever seen to contain a correct sized supercoil comigrating with the input form (Fig. 4.5 , lane 11, Fig. 4.4. lane 2, Fig. 3.3 lane 1). These occurrences were isolated events, for example in Fig.4.5 only 1/12 of the pΔAT153/BPV$_I$ or II

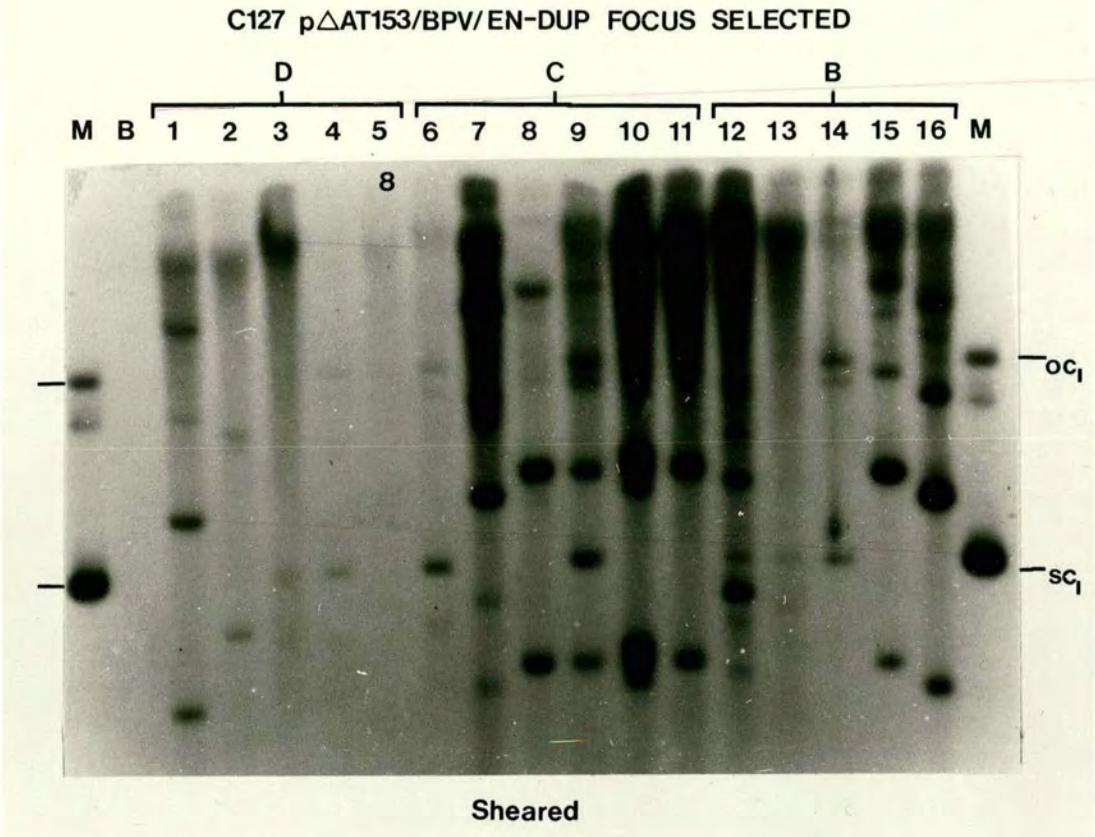
FIGURE 4.7. pΔAT153/BPV$_I$/EN-DUP-D, C or B (as indicated) DNAs in C127 focus selected cell lines.

Cellular DNA (5μg) was sheared by passage through a 25 gauge syringe. Samples were electrophoresed through a 0.7% agarose gel, transferred to nitrocellulose and hybridised to a nick translated probe, BPV-1. All these cell lines were derived by calcium phosphate precipitation.

Samples:-

M; 10 copy equivalent of undigested pΔAT153/BPV$_I$/EN-DUP-D (11.3Kb.) input plasmid in 5μg of sheared 3T3 DNA.
B; 5μg of sheared 3T3 DNA.

1; DNA extracted from C127 pΔAT153/BPV$_I$/EN-DUP-D Clone 1. 2; D Clone 2. 3; D Clone 3. 4; D Clone 4. 5; D Clone 5. 6; pΔEN-DUP-C Clone 1. 7; C Clone 2. 8; C Clone 3. 9; C Clone 4. 10; C Clone 5. 11; C Clone 6. 12; pΔEN-DUP-B Clone 1. 13; B Clone 2. 14; B Clone 3. 15; B Clone 4. 16; B Clone 5.


C127 pΔAT153/BPV/EN-DUP FOCUS SELECTED
D
C
B
M B 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M
8
OC I
SC I
Sheared

transformant DNAs (transformed with vectors similar to the pΔAT153/BPV$_I$/EN-DUP vector but without a duplicated enhancer) contained a supercoiled form of the correct size. In the present transformations a total of 7 out of the 16 DNAs examined are seen to contain a supercoiled of the correct size. It is possible that all these represent dimeric rearranged/deleted forms, but there seems to be little difference in the mobility of this form in these 7 independent transformants. It is unlikley that random deletions would generate the same sized molecule in all 7 cell lines, unless, of course, a specific deletion event was favoured due to the arrangement of sequences in all three vector orientations (pB, pC and pD). These DNAs have not as yet been analysed by restriction enzyme cleavage, but, it is likely that the results of such an analysis would be complex due to the several different forms present in each cell line. The analysis of C127 transformants generated by the microinjection of either pC or pD gave similar results (data not shown). An attempt to recover these correct sized forms into competent E.coli by transformation with circular DNA purified from the DNAs of cell lines, and selection of ampicillin resistant colonies, has been made and the results are presented in Chapter 6.

4.8. Discussion

This Chapter describes the construction of a series of simple BPV-1 containing vectors, which consisted of a bacterial plasmid linked to BPV-1 sequences. These were tested for their replication as unrearranged monomeric extrachromosomal molecules when introduced into mouse C127 cells, the eventual aim being to reinsert a marker selectable in mammalian cells such as HSV-TK, TK-Neo or SV_2 neo (see Chapter 1 for discussion and Fig.2.9).

The fact that the only episomal forms obtained with both pBR328/BPV_I and pAT153/BPV_I were deletants suggested, on comparison of the results of Sarver *et al* (1982) and Binetruy *et al* (1982), that a small fragment of bacterial plasmid DNA, (i.e. the HindIII/BamHI 344bp fragment) may be inhibitory to replication as intact circular forms. To test this the HindIII/BamHI 344bp fragment was deleted from pBR328 and pAT153 to give the vectors pΔBR328/BPV_I and pΔAT153/$BPV_{I\&II}$. In addition one copy of the inverted repeat of pBR328 was also deleted giving pΔIR/BPV_{II}. The use of both pBR328 and pAT153 controls for any inhibitory effects that plasmid size or the additional chloramphenicol resistance marker in pBR328 might have on the replication of pBR328-BPV-1 hybrid vector. Disappointingly, none of these vectors were found to replicate as monomeric intact circular forms in C127 transformants.

Sarver et al (1982) had suggested that the SV40 poison sequences of pBR322 are responsible for the rearrangements seen on introduction of the pBR322-BPV-1 hybrid molecule into C127 cells since the 'SV40 poisonless' plasmid, pML2d, allowed episomal replication of unrearranged pML2d-BPV-1 hybrid molecules in C127 cells. But, neither pBR328 or pAT153 contain sequences inhibitory to the replication of SV40, since both pBR328-SV40 and pAT153-SV40 recombinant molecules can replicate in monkey cells (Rigby, 1982). A series of plasmids pΔAT153/BPV_I/EN-DUP B, C and D were therefore constructed taking into account the arrangement of control regions on the normal episomally replicating BPV-1 genome. In these vectors the BPV-1 enhancer was duplicated at a position which would place it 2.5kb 5' to the region where the viral origin of replication, a DN'ase hypersensitive site and the putative viral transcriptional promoter map. This is the normal relative position for the enhancer in the circular BPV-1 genome. The enhancer was placed in either the normal orientation (pC) or the reverse orientation (pD), and, as a control, it was duplicated as an inverted repeat adjacent to the original enhancer (pB).

In the resulting transformants only 7 of the 16 DNAs examined had a band which might have represented intact supercoiled vector detected. Two such cell lines were generated upon transformation with either pC or pD, but three resulted from transformation with pB. It was

expected that perhaps pC and pD would replicate as intact vectors but not pB, which was expected to behave in an identical manner to $p\Delta AT/BPV_{I \text{ or } II}$. The results were not as clear cut, since the correct size molecules were only present as a minor form, the majority of the transforming sequences were present as deleted monomeric supercoiled and/or higher molecular weight forms. The fact that pB transformants also contained a correctly sized band suggests that the effect of having two adjacent enhancers might be additive and thus the inhibitory effects of large distances between the enhancer and the promoter could be overcome.

Whether or not these 'enhancer duplication' transformants do contain truly intact monomeric circular forms has yet to be demonstrated, but recovery experiments are presented later (Chapter 6). About half of the cell lines contained only deleted episomal forms and higher molecular weight material, and these rearranged forms were also present in DNAs containing the intact vector. This suggests that the vectors pB, pC and pD are still not functioning in the desired manner. The reason for this is not known, but may stem from the use of a BPV-1 variant intolerant to the presence of additional vector sequences or from the cell culture system used (i.e. an effect of the medium, serum, transformation system, etc.). To test this possibility, a variety of vectors have been obtained from other laboratories containing a different BPV-1 isolate. These

vectors have been reported to be maintained as intact, monomeric open and supercoiled circles when introduced into mouse C127 cells. Some of these vectors incorporate the use of a selectable marker and the results obtained are presented in the next Chapter.

CHAPTER 5

EXTRACHROMOSOMAL MAINTENANCE OF VECTORS OBTAINED FROM OTHER SOURCES AND INSERTION OF A SELECTABLE MARKER

5.1. Introduction

In Chapter 1 (1.9) a set of properties were listed which described the behaviour and characteristics that the ideal BPV-1 vector should possess. As has been seen in Chapters 3 and 4, none of the vectors constructed in the work presented so far could be said to conform with all of these properties. The only vectors which have been seen to give rise to small amounts of monomeric forms in transformed cell lines are the pΔAT153/BPV_I/EN-DUP/B/C/D vectors and then in only less than half of the cell lines examined.

To investigate whether the cell culture or transformation systems used were at fault in these experiments, the vector pBRd BPV-β_1 obtained from D. DiMaio (Harvard University) was utilised. DiMaio et al (1982) had shown that this vector replicates as an extrachromosomal unrearranged form in mouse C127 cells. In addition, the vector $pCGBPV_{9\Delta B5}$ was obtained from P. Matthias. This vector contains a dominant selectable marker (Neo) which confers resistance to the compound G418 in eukaryotic cells (see Chapter 1). This vector, and the one from which it was derived ($pCGBPV_9$), replicates, in most cases, as unrearranged extrachromosomal forms in G418 selected cell lines (P. Matthias, pers. comm. and P. Matthias et al, 1983). Analysis of cell lines transformed with these vectors is presented below. Recently, a similar vector pdBPV-MMTneo

has been obtained from P. Howley, which has also been shown to be maintained as a stable extrachromosomal element in mouse C127 cells selected for resistance to G418 (Law et al, 1983). This vector, together with pML2d-BPV-1 (Sarver et al, 1982) which was also obtained recently from P. Howley, has been tested under our conditions of transformation of C127 cells.

The presence of a selectable marker in a BPV-1 vector should allow it to be introduced into a wider range of host cells and this would conform with one of the properties that the ideal BPV-1 vector should exhibit. In Chapter 3, an unsuccessful attempt was made to include the HSV-TK selectable gene in a BPV-1 vector, neither TK^+ L cell transformants or focus selected C127 cell transformants were found to contain the vector as an intact extrachromosomal form. In this Chapter, some of the results obtained on insertion of the HSV-TK or SV_2neo selectable markers into some of the deletion type vectors constructed in Chapter 4 are also mentioned.

5.2. Transformation of C127 cells with pBRd BPV-β_1

The vector pBRd BPV-β_1 was constructed by insertion of the 69% HindIII/BamHI fragment of BPV-1 DNA between the HindIII and BamHI sites of pBRd (this is essentially the same as pML2 see Fig. 4.3). This results

in the deletion of the small 344bp pBRd HindIII/BamHI fragment, generating pBPV-HII. A 7.6kb HindIII fragment of the human β-globin gene cluster was inserted into the HindIII site of pBPV HII to generate pBRd BPV-β_1 Fig. 2.7 (DiMaio et al, 1982).

On introduction of this vector, pBRd BPV-β_1, into mouse C127 cells by the calcium phosphate precipitation method using carrier DNA, it was found by DiMaio et al (1982) that the transformation frequency was about 500-fold more than that obtained with intact pBPV-HII (no β-globin). In cell lines originating from single foci the vector was found to be present mainly as an intact monomeric extrachromosomal circular form, which comigrated with the input plasmid. It was claimed that no diffuse, high molecular weight forms were detected similar to those seen in some other BPV-1 transformants (Sarver et al, 1981; Law et al, 1981). Restriction enzyme cleavage with SalI (1 site within pBrd BPV-β_1), converted all hybridising material to a single band, which comigrated with the 16kb linear input DNA. Nevertheless, DiMaio et al (1982) point out that their results do not exclude the possibility that a small proportion of the vector DNA had integrated into high molecular weight chromosomal DNA. Restriction enzymes with multiple sites in the vector did not reveal extensively rearranged forms of the vector in these cell lines. One cell line did contain additional bands homologous to the input vector and were found to

represent deleted forms of the vector. Apparently deleted vector forms were also detected after prolonged passage of other cell lines which contained unrearranged forms upon initial examination. It was found that unrearranged forms of the plasmid could be recovered into competent E.coli as ampicillin resistant colonies by transformation with low molecular weight DNA extracted from the early passage cell lines (DiMaio et al, 1982).

From the above results of DiMaio et al (1982), it appeared that this vector satisfies many of the properties desired of a BPV-1 vector. The pBRd BPV-β_1 vector was therefore obtained from D. DiMaio and used to test the cell culture and transformation systems used here. pBRd BPV-β_1 was introduced into mouse C127 cells by either calcium phosphate precipitation or nuclear injection, and the results obtained are presented below.

5.3. Analysis of Cellular DNA in pBRd BPV-β_1 transformants

Total cellular DNA was extracted from six C127 cell lines derived from independent foci by the precipitation procedure and four C127 cell lines derived from independently injected cells transformed with pBRd BPV-β_1. Sheared samples of these DNAs were analysed by blot hybridisation experiments using the complete vector,

pBRd BPV-β_1, as a probe. The results, in Fig. 5.1, show that all the transformed cell lines contained the vector as intact, monomeric, extrachromosomal supercoiled and open circular forms (input plasmid DNA, lane M). In addition to these forms there was clearly a large amount of higher molecular weight material detectable as a diffuse smear. There were also other specific bands, which possibly corresponded to the dimeric/multimeric forms or to linear form(s) of the vector DNA. In one cell line a minor, presumably deleted circular form, was detected (lane 9, marked by arrow). No further analysis was carried out on these cell lines, but the results of an attempt to recover intact vector into competent E.coli are presented in Chapter 6.2.A.

The results obtained with this pBRd BPV-β_1 vector are very similar to those of DiMaio et al (1982) detailed above. Intact, monomeric circular forms were present in all cell lines regardless of the method of introducing the vector DNA. Like DiMaio et al (1982) one cell line was seen to contain a minor deleted circular form. Surprisingly, however, high molecular weight material was detected in all cell lines regardless of the method of introducing the DNA. This high molecular weight material may represent vector DNA which has integrated into the host genome. On closer examination of the results of DiMaio et al (1982), this high molecular weight material was also present as a very faint smear. Thus, the results obtained with pBRd BPV-β_1

FIGURE 5.1. pBRd/BPV-β_1 DNA in C127 focus selected cell lines.

Cellular DNA (5μg) was sheared by passage through a 25 gauge syringe. Samples were electrophoresed through a 0.7% agarose gel, transferred to nitrocellulose and hybridised to a nick translated probe, pBRd/BPV-β_1.

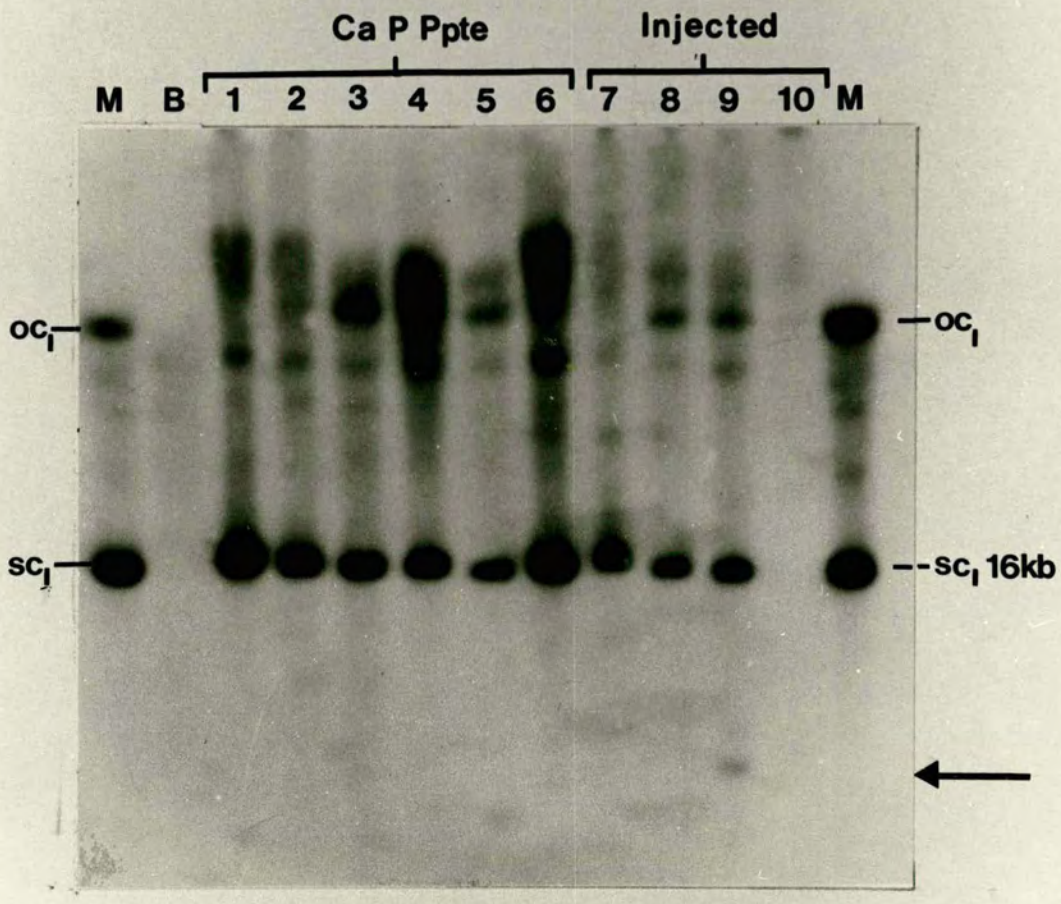
Samples:-

M; 10 copy equivalent of undigested pBRd/BPV-β_1 input plasmid in 5μg of the sheared Ltap207 DNA. B; 5μg of sheared Ltap207 DNA.

1; DNA extracted from C127 pBRd/BPV-β_1 Clone 1. 2; Clone 2. 3; Clone 3. 4; Clone 4. 5; Clone 5. 6; Clone 6. 7; Ij 4A. 8; Ij 4B 9; Ij 6A. 10; Ij 8A.

Samples 1-6 were derived by calcium phosphate precipitation.
Samples 7-10 were derived by microinjection.

C127 pBRd/BPV-β FOCUS SELECTED



tend to suggest that the cell culture and transformation (precipitation or injection) systems used in this laboratory can directly reproduce the results of other workers who use BPV-1 based vectors and who may employ a slight variation of the methods for introducing the vector into C127 cells (e.g. carrier DNA present, DMSO shock).

A similar vector, pBR328/BPV$_{69\%}$/TK/Hβ_I^8 was constructed which contained the HSV-TK PvuII fragment, the BPV 69%T fragment and the human β-globin 7.6kb HindIII fragment inserted into pBR328. Introduction of this vector into mouse Ltap207 cells by precipitation and selection for TK$^+$ colonies yielded cell lines which only contained the vector in a high molecular weight form (results not shown). Similar results were found by D. DiMaio (pers. comm.) on insertion of the HSV-TK gene into pBRd BPV-β_1. In one NIH3T3 (which has similar contact-inhibited growth properties to C127 cells) focus selected cell line transformed with pBR328/BPV$_{69\%}$/TK/Hβ_I^8, the vector sequences were found to be present as deleted circular forms and as high molecular weight forms. No intact monomeric circles could be detected (data not shown). Thus it would appear that the HSV-TK gene is inhibitory to the replication of vectors containing BPV-1 as intact circular forms in mouse fibroblast cell lines in agreement with the results found in Chapter 3 and those of Lusky et al (1982; 1983).

5.4. Transformation of C127 cells with $pCGBPV_{9\Delta B5}$ - a vector containing the TK-Neo dominant selectable marker

The vector $pCGBPV_{9\Delta B5}$ (Fig. 2.8), constructed by P. Matthias, contains the complete linear BPV-1 HindIII fragment, a ColE1 replication origin from pHSG262 (Hashimoto-Gotah and Inseling, 1979) [as opposed to the pBR322 replication origin which was derived from pMB1, a 'ColE1 like' plasmid (Bolivar et al, 1976; 1977a)], and the APH(3')II gene from the bacterial transposon Tn5 which confers resistance to kanamycin in bacteria. This gene is under the control of the eukaryotic HSV TK promoter as well as a prokaryotic promoter from pBR322 (TK-Neo Fig.2.9). This arrangement allows for the selection of kanamycin resistant colonies in E.coli and G418 resistant colonies in eukaryotic cells (see Chapter 1.4). In addition the vector contains the cos site of bacteriophage λ which allows the use of the in vitro packaging reaction to clone large DNA fragments (Hohn and Murray, 1977). $pCGBPV_{9\Delta B5}$ is a derivative of the $pCGBPV_9$ constructed by P. Matthias et al (1983), but it differs only in that the BamHI site internal to the BPV-1 HindIII insert has been destroyed.

Matthias et al (1983) introduced the vector $pCGBPV_9$ into mouse C127 cells by the calcium phosphate precipitation technique in the presence and absence of calf thymus carrier DNA, using the same amount of vector

DNA in both experiments. Analysis of undigested DNA, extracted from G418 resistant C127 transformants, by blot hybridisation experiments revealed the presence of intact monomeric, supercoiled (FI) and open circles (FII) in all cell lines (with and without carrier DNA). Only one cell line out of eleven was found to contain a smaller extrachromosomal form. All of the eleven $G418^R$ clones examined also contained a complex of slow migrating DNA, which it was suggested might represent dimeric supercoiled and open circular forms, and high molecular weight integrated DNA. Restriction enzyme analysis revealed the presence of unexpected fragments in one or two of the five cell lines examined in detail, and these may represent junction fragments of an integrated structure or rearranged extrachromosomal forms present at a low copy number. Matthias et al (1983) also demonstrated that $pCGBPV_9$ could be rescued fom $G418^R$ C127 cells by transformation of competent E.coli to kanamycin resistance with total DNA from ten different transformants. This confirmed the extra-chromosomal nature of some of the vector sequences.

There are three further points of interest arising from the results of Matthias et al (1983). Firstly, mouse C127 cells transformed with the $pCGBPV_9$ vector and selected for focus formation apparently resulted in cell lines which did not exhibit resistance to G418. Secondly, use of a vector $pCGBPV_7$ (where transcription of BPV-1 is in the same direction as that of the TK-Neo gene i.e.

the relative orientation of the two genes is opposite to that in $pCGBPV_9$ - see Fig. 2.8) resulted in $G418^R$ cell lines where only 1 out of 7 contained intact, monomeric cirular vector. All the other clones contained the vector DNA migrating as a high molecular weight species. Thirdly, other BPV-1/TK-Neo plasmids, which contained the bacterial ampicillin gene and replication origin of pML2 (i.e. Col E1A) but no λ cos sequences, apparently gave similar results to those found with $pCGBPV_7$. Only a small minority of the G418 resistant colonies contained unrearranged extra-chromosomal monomeric plasmid.

Thus, the vector $pCGBPV_9$ seemed to satisfy most (1-6) of the properties required of a BPV-1 vector as presented in Chapter 1. The vectors $pCGBPV_9$ and $pCGBPV_{9\Delta B5}$ were obtained from P. Matthias. Since $pCGBPV_{9\Delta B5}$ contains only a single BamHI site which we hoped to utilise in future work, and since it behaves in the same manner as $pCGBPV_9$ (P. Matthias, pers. comm.), it was used to test our cell culture and transformation systems. The results obtained on the introduction of this vector into C127 and Ltap207 cells is presented below.

5.5. Analysis of Cellular DNA in $pCGBPV_{9\Delta B5}$ (a) G418 and (b) Focus selected cell lines

The vector $pCGBPV_{9\Delta B5}$ was introduced into mouse C127 cells by both the precipitation and nuclear injection methods and either selected for G418 resistant colonies or for morphologically transformed foci.

a) G418 selected cell lines:-

On selection for resistance to G418, colonies first appeared containing flat non-transformed (morphologically) cells. After cloning, growth and further passaging, all cell lines eventually became morphologically transformed. Similar observations have been made by Matthais et al (1983); Law et al (1983) and Lusky and Botchan (1984), with BPV-$G418^R$ vectors.

Analysis of sheared total cellular DNA extracted from C127 cells transformed to G418 resistance with $pCGBPV_{9\Delta B5}$ by blot hybridisation is presented in Fig. 5.2.A. This analysis indicates that vector sequences were present only as high molecular weight forms in most of those $G418^R$ lines resulting from transformation by the precipitation method. No monomeric extrachromosomal circular forms of the vectors could be detected in 4 of these cell lines (Figure 5.2.A, lanes 1-4). In one $G418^R$ cell line (figure 5.2.A, lane 5), resulting from calcium phosphate precipitation transformation, a

FIGURE 5.2. $pCGBPV_{9\Delta B5}$ DNA in C127

(A) G418 selected cell lines,

(B) Focus selected cell lines.

Cellular DNA (5µg) was sheared by passage through a 25 gauge syringe. Samples were electrophoresed through a 0.6% agarose gel, transferred to nitrocellulose and hybridised to a nick translated probe, $pCGBPV_{9\Delta B5}$.

Samples:-

A. (Top opposite) G418 selected cell lines. M; 10 copy equivalent of undigested $pCGBPV_{9\Delta B5}$ input plasmid in 5µg of the sheared Ltap207 DNA. B; 5µg of sheared Ltap207 DNA. 1; DNA extracted from C127 $G418^R$ $pCGBPV_{9\Delta B5}$ Clone 1. 2; Clone 2. 3; Clone 3. 4; Clone 4. 5; Clone 6. 6; pool. 7; Ij 69A. 8; Ij 71A 9; Ij 71B. 10; Ij 72A. 11; Ij 72B. 12; Ij 73A. 13; Ij 74A. 14; Ij 75A.

Samples 1-6 were derived by calcium phosphate precipitation.

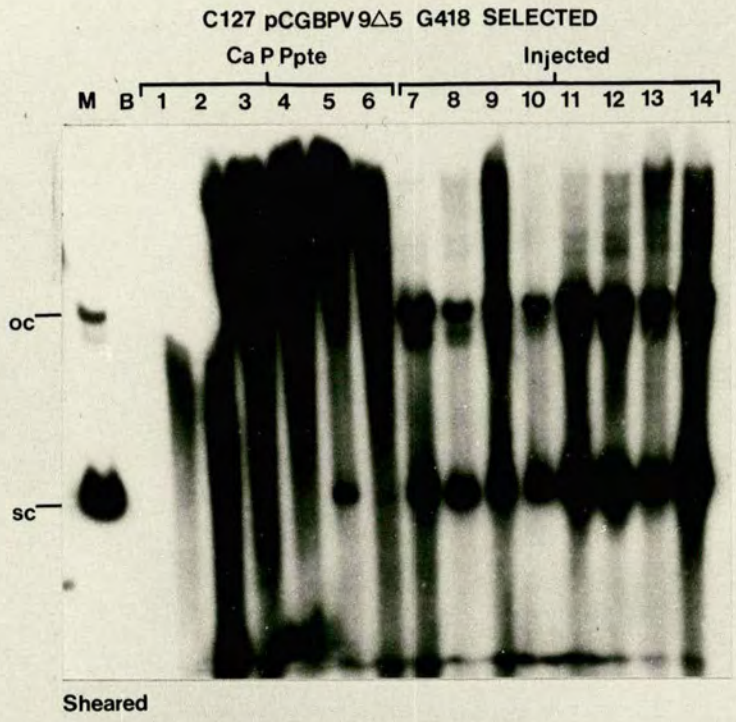
Samples 7-14 were derived by microinjection.

B. (Bottom opposite) Focus selected cell lines. M; 10 copy equivalent of undigested $pCGBPV_{9\Delta B5}$ input plasmid in 5 µg of the sheared Ltap207 DNA. B; 5µg of sheared Ltap207 DNA. 1; DNA extracted from C127 $pCGBPV_{9\Delta B5}$ Focus Clone 1. 2; Clone 2. 3; Clone 3. 4; Clone 4. 5; Clone 5. 6; Clone 6. 7; Clone 3 (2nd prep.). 8; Clone 6 (2nd prep.). 9; Ij 59A. 10; Ij 62A. 11; Ij 62B. 12; Ij 63A. 13; Ij 64A. 14; Ij 64B. 15; Ij 65A.

Samples 1-8 were derived by calcium phosphate precipitation.

Samples 7-15 were derived by microinjection.

DNA samples 3 and 7 were derived from the same cell line, similarily for samples 6 and 8.


C127 pCGBPV 9△5 G418 SELECTED
Ca P Ppte
Injected
M B 1 2 3 4 5 6 7 8 9 10 11 12 13 14
oc
sc
Sheared


C127 pCGBPV 9△5 FOCUS SELECTED
Ca P Ppte
Injected
M B 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M
oc
sc
Sheared

band can be seen which comigrates with the monomeric supercoiled form of the input $pCGBPV_{9\Delta B5}$ vector (labelled SC in marker lane M). Lane 6 of Figure 5.2.A contained sheared total cellular DNA extracted from the mixture of $G418^R$ colonies which remained after picking 6 independent colonies (i.e. it is a pool of many different transformation events). In this sample only a minor proportion of the hybridising DNA was present as an intact monomeric supercoil. This suggests that in the cell population of $G418^R$ cells derived by the precipitation method, only a small number of colonies contained unrearranged monomeric supercoiled $pCGBPV_{9\Delta B5}$, or all colonies contained only a small amount of the intact vector. In contrast, on analysis of 8 independent $G418^R$ cell lines resulting from microinjection of the vector, all were seen to contain a major proportion of vector sequences as monomeric supercoiled and open circular forms (Fig.5.2.A. lanes 7-14). Other bands were detected, but these presumably correspond to multimeric forms of the vector. The amount of diffuse, high molecular weight material would appear to be negligible in most of these clones, except perhaps in lanes 9, 13 and 14.

On digestion of these same DNAs with the restriction endonuclease BamHI (1 cleavage site in the vector, Fig. 2.8) the DNAs derived from the precipitation experiment were found to contain a major band of 11.3kb, equivalent to the linear form of the input vector (data not shown).

But in most cases, additional, unexpected bands were also detected which probably represent either junction fragments generated from integrated copies or deleted forms present in the high molecular weight entity. In the $G418^R$ clones derived by microinjection of the $pCGBPV_{9\Delta B5}$ vector, BamHI digestion generated only the linear form of the vector (i.e. a 11.3kb band) no other bands were detected (data not shown). These results suggest that the $pCGBPV_{9\Delta B5}$ vector replicates mainly as an unrearranged monomeric circular form when introduced into C127 by microinjection. In contrast, introduction by the precipitation method results in the aggregation of rearranged and intact forms of the vector into a high molecular weight complex which might be integrated into the host genome.

Further analysis of the DNAs in two of the $G418^R$ cell lines (clone 6 - calcium precipitation-derived in lane 5, and clone 74A - injection-derived in lane 13, of Figure.5.2) was carried out by digestion with the ATP dependent DNase, ExoV. This enzyme digests any linear forms to oligo-nucleotides of average size 5.5 base pairs. (Anai et al, 1970, see Chapter 2.10). Figure 5.3 shows undigested and ExoV treated total cellular DNA. The amount of high molecular weight material seen in the undigested sample of the precipitation-derived transformant is substantially reduced upon digestion with ExoV (lanes 1^- and 2^+, undigested and digested, respectively). Faint bands which were masked by

FIGURE 5. 3. Exo V digestion of C127 $pCGBPV_{9\Delta B5}$ $G418^R$ clones.

Cellular DNA (5µg) from the transformants shown in Fig.5.2A lanes 5 and 13 were either untreated (- : lanes 1 and 3 respectively) or were digested with an excess of the ATP-dependent DNAase Exo V (+ : lanes 2 and 4). Samples were electrophoresed through a 0.6% agarose gel, transferred to nitrocellulose and hybridised to a nick translated probe, BPV-1.

Samples:- M; 10 copy equivalent of BamHI linearised BPV-1 (7.945Kb) and HpaI linearised $pAT153/BPV_I$ (11.6Kb) and $pBR328/BPV_I/TK_2$ (15.0Kb).

m; 10 copy equivalent of HpaI linearised $p\Delta BR328/BPV_I$ (12.5Kb).

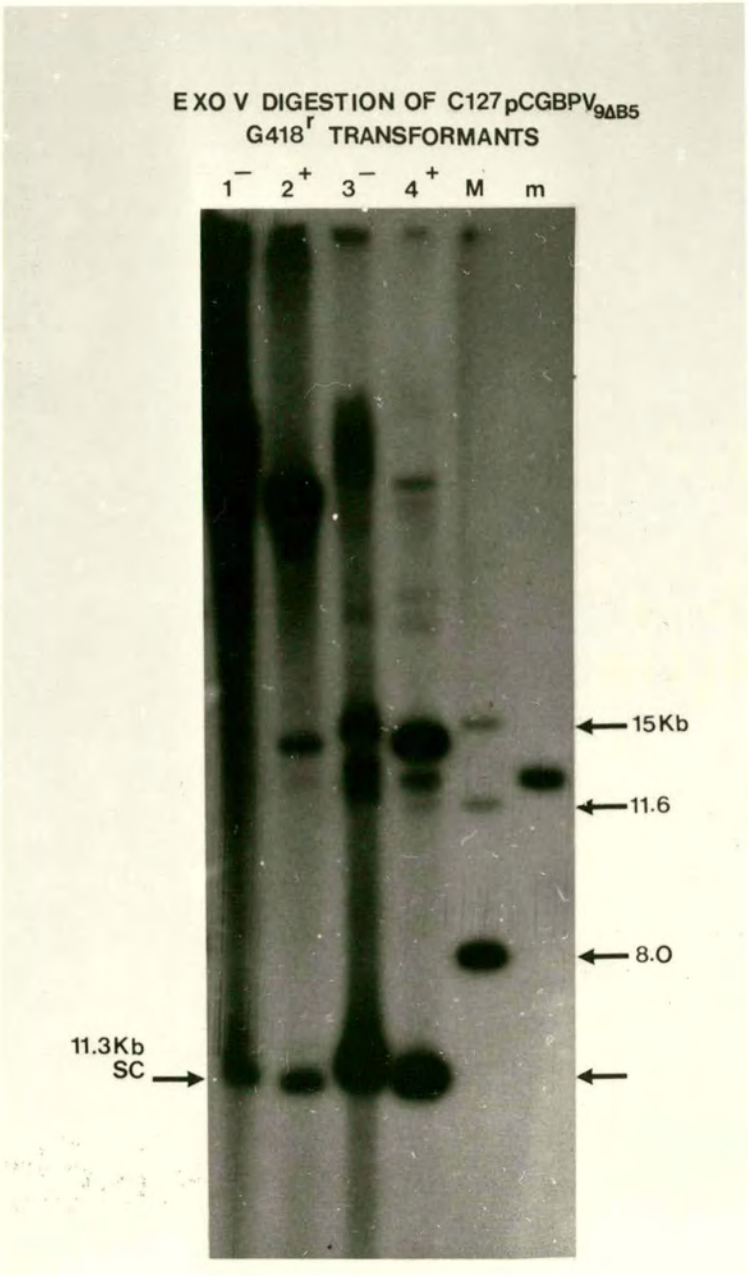
1; Untreated DNA extracted from C127 $G418^R$ $pCGBPV_{9\Delta B5}$ Clone 6.

2; Exo V digest of DNA extracted from C127 $G418^R$ $pCGBPV_{9\Delta B5}$ Clone 6.

3; Untreated DNA extracted from C127 $G418^R$ $pCGBPV_{9\Delta B5}$ Ij 74A.

4; ExoV digest of DNA extracted from C127 $G418^R$ $pCGBPV_{9\ B5}$ Ij 74A.

Lower arrows : indicate the monomeric supercoiled form of the 11.3Kb $pCGBPV_{9\Delta B5}$ plasmid.


EXO V DIGESTION OF C127 pCGBPV9ΔB5
G418r TRANSFORMANTS
1− 2+ 3− 4+ M m
15Kb
11.6
8.0
11.3Kb
SC

hybridisation to the high molecular weight smear can now be seen. Analysis of the injection-derived transformant also shows that the small amount of heterogeneous high molecular vector DNA is removed upon digestion with ExoV (Fig. 5.3 lanes 3^- and 4^+, undigested and digested, respectively). This result shows that the high molecular weight species present in these $pCGBPV_{9\Delta B5}$ $G418^R$ C127 transformants is accessible to the enzyme and therefore is a linear form of the vector DNA. It may, therefore, be derived from broken large circular forms or from integrated copies of the vector DNA.

b) Focus selected cell lines:-

Upon introduction of $pCGBPV_{9\Delta B5}$ by either precipitation or injection, some cells were selected for their ability to form foci in the absence of G418. The resulting foci were picked and sheared total cellular DNA was analysed by blot hybridisation. The results in Fig. 5.2.B. reveal that the six independent transformants resulting from the precipitation method (lanes 1-6; lanes 7 and 8 are DNA preparations made from different samples of the same cell lines analysed in lanes 3 and 6 respectively) contained the vector DNA as a deleted supercoiled form. Comparing these results with those presented in Chapters 3 and 4 suggests that these extrachromosomal forms are probably about 7-9kb in size and retain most, or all, of the BPV-1 sequences with only a small quantity of the plasmid and TK-neo gene

sequences. Matthias et al (1983) reported similar results in that many foci did not exhibit resistance to G418. In the three focus transformants derived by microinjection (only 3 very small foci were found to be positive), the vector sequences appeared to be present as intact monomeric circular forms in two of them (Fig. 5.2.B lanes 10 and 15), since they contained two bands which comigrated with the input vector DNA (shown in lanes M). The other injection-derived focus transformant (lane 12) was similar to those seen in the precipitation-derived cell lines, in that it contained a smaller, deleted form of the vector. However, the injection deletant cell line (lane 12) differs from the precipitation cell lines in that no heterogeneous high molecular weight material was detected. None of these focus selected cell lines were examined by enzymatic cleavage.

The results obtained with this $pCGBPV_{9\Delta B5}$ vector suggest that the method of introducing the vector into the cells influences the state of the vector in resulting $G418^R$ or focus selected cell lines. On the basis of the above experiment, it would appear that microinjection avoids both the aggregation of the $pCGBPV_{9\Delta B5}$ vector into the high molecular weight complex seen in $G418^R$ cell lines, and the frequency of deletions suffered in focus selected cell lines, derived by the precipitation method. The use of direct microinjection of DNA into the nucleus avoids the degradative events which are thought

to occur when the precipitated DNA passes through the cell cytoplasm (Loyter et al, 1982a and b) and much of this degradation may be attributable to passage through lysosomes (Luthmann and Magnusson, 1983).

The above explanation does not explain why an almost identical vector, $pCGBPV_9$, when introduced by Matthias et al (1983) by the precipitation procedure gives rise to $G418^R$ colonies, the majority of which seemed to contain mainly intact extrachromosomal copies of the vector. Nor does it explain why the introduction of the vector pBRd BPV-β_1 (see Section 5.3) resulted in intact copies of the vector in both precipitation- and injection-derived cell lines. Perhaps the culture and/or transformation conditions used in the various transformation experiments differs due to unknown entities. These might include variability in foetal calf serum batch quality or variability in the DNA precipitate, so that the DNA is subjected to less degradation in some experiments than it is in others. Since injection of pBR BPV-β_1 and $pCGBPV_{9\Delta B5}$ both gave intact monomeric vector present in the transformants, microinjection would seem to be the method of choice for introducing these vectors. However, the precipitation procedure is preferrable when the expected transformation frequency is very low (i.e. $<1 \times 10^{-2}$).

$pCGBPV_{9\Delta B5}$ has also been introduced into Ltap207 cells by both methods with selection for

$G418^R$ colonies. In contrast to the results found in C127 cells, no monomeric extrachromosomal forms were found in either precipitation or injection derived transformants, but all vector homologous sequences were present as a high molecular weight form (data not shown). This result was reminiscent of the situation in LTK^+ transformants containing the pBR328/BPV/TK_2 vector in Chapter 3. Together the results tend to suggest that the L TK^- cell line used (Ltap207) lacks some cellular factor required for the extrachromosomal replication of BPV-1 DNA and which may be required to interact with the BPV-1 enhancer to activate viral expression. The existence of such factors in cultured cells is suggested by the work of Scholer and Gruss (1984).

5.6. Analysis of G418 resistant pΔBR328/BPV/Neo Transformants

A vector similar to that of Matthias et al (1983) was constructed in the following way. The SV_2Neo transcription unit (Southern and Berg, 1982, see Fig. 2.9) contained in a 2.6kb BamHI fragment was inserted into one or other of the BamHI sites of pΔBR328/BPV_1 (see Fig. 2.4) in both orientations. This generated the vectors pΔBR328/BPV_I/$Neo_{I,II,III\&IV}$, where I→IV refers to the different orientations of SV_2Neo (Fig. 2.4). Equal quantities of each vector (I,II,III&IV) were

then mixed and used to form one calcium phosphate precipitate, which contained a total concentration of vector DNA of 20µg/ml (5µg/ml of each vector). This precipitate was then placed onto C127 cells and transformed colonies selected by their ability to grow in G418. No individual $G418^R$ colonies were picked, but whole $G418^R$ cell populations resulting from duplicate transformation experiments were expanded and combined into one cell line, which therefore contained a heterogenous population of cells transformed with any combination of the four vectors. Total cellular DNA was extracted from this mixed population of $G418^R$ cells and analysed by blot hybridisation experiments.

On shearing of either 5 or 8 µg of this DNA, (lanes 1 and 2, respectively, Fig. 5.4) no extrachromosomal monomeric forms of the input DNA could be detected by hybridisation. All hybridising material was present as a high molecular weight species, which, on comparison with the λ marker track (not shown) in the original ethidium bromide stained gel photograph, must be greater than 40kb. Lane 3 shows a HindIII digest of this total cellular DNA. Each vector orientation gives two characteristic bands upon HindIII digestion. These are shown in lanes 4-7 which contains HindIII digests of orientations I, II, III & IV, respectively, mixed with 5µg of L cell DNA. All the expected bands generated upon HindIII digestion of all vectors are detected in the "mixed" cellular DNA. This suggests that the vector DNAs

FIGURE 5.4. MIXTURE OF $p\Delta BR328/BPV_I$/NEO I, II, III, AND IV.

Cellular DNA (5µg lanes 1 and 3, 8µg lane 2) was sheared by passage through a 25 gauge syringe or were digested with a fivefold excess of HindIII. Samples were electrophoresed through a 0.8% agarose gel, transferred to nitrocellulose and hybridised to a nick translated probe, $pBR328/BPV\text{-}1/TK_2$.

Samples:- M; 10 copy equivalent of undigested $pBR328/TK_2/BPV_I$ (15.0Kb approx. same size as input vectors) in 5µg of Ltap207 DNA.

1; Sheared 5µg sample of DNA from C127 $p\Delta BR328/BPV_I$/NEO I,II,III & IV $G418^R$ mixed cell population .

2; Sheared 8 g sample of DNA from C127 $p\Delta BR328/BPV_I$/NEO I,II,III & IV $G418^R$ mixed cell population .

3; HindIII digested sample of DNA from C127 $p\Delta BR328/BPV_I$/NEO I,II,III & IV $G418^R$ mixed cell population .

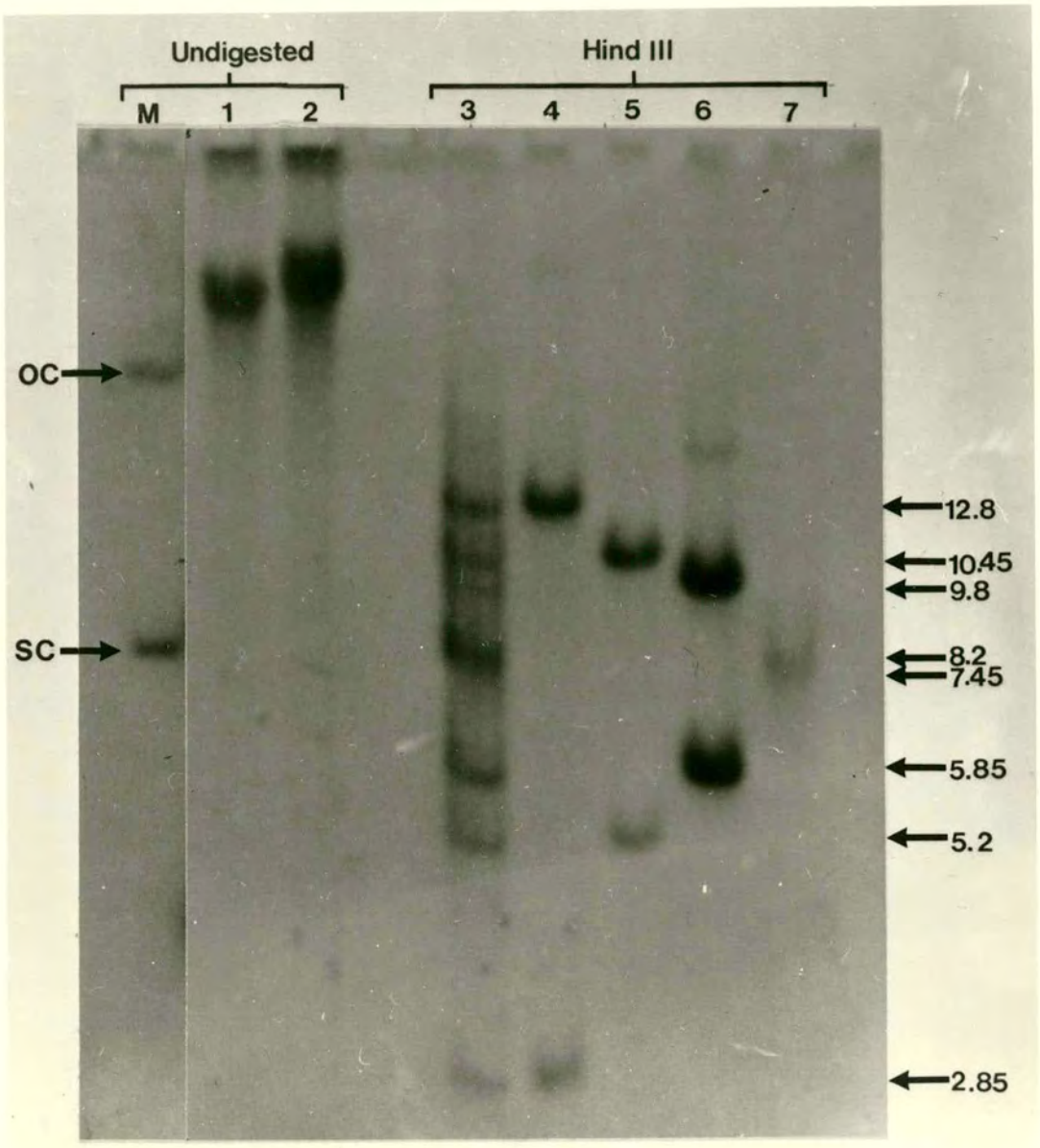
4; HindIII digest of 10 copy equivalent of $p\Delta BR328/BPV_I$/NEO I (15.0Kb approx.) in 5µg of Ltap207 DNA.

5; HindIII digest of 10 copy equivalent of $p\Delta BR328/BPV_I$/NEO II (15.0Kb approx.) in 5µg of Ltap207 DNA.

6; HindIII digest of 10 copy equivalent of $p\Delta BR328/BPV_I$/NEO III (15.0Kb approx.) in 5µg of Ltap207 DNA.

7; HindIII digest of 10 copy equivalent of $p\Delta BR328/BPV_I$/NEO IV (15.0Kb approx.) in 5µg of Ltap207 DNA.

Samples 4-7 serve as markers for orientations I, II, III, or IV in HindIII digests of this DNA derived from a mixed poulation of $G418^R$ cells.


Undigested
Hind III
M
1
2
3
4
5
6
7
OC
SC
12.8
10.45
9.8
8.2
7.45
5.85
5.2
2.85

were organised into oligomeric, high molecular weight structures, which could have been integrated into the host chromosome. No bands of an unexpected size were detected which would be indicative of an integration event, but since this is a mixed cell population (from possibly 1000 $G418^R$ colonies), such single copy bands would not be detected as they would be present at 1×10^{-3} copies per genome equivalent in this DNA. The background smear seen mainly between the 5.2kb and 12.8kb bands in lane 3 could be due to hybridisation to many different junction fragments representing many different integration events in different cells. Alternatively, this smear may be a product of digestion of many rearranged copies of the vector which might be present in a high molecular weight, extrachromosomal element. Of course, it is possible that both events have occurred in the same or different cells in the population.

The vector $p\Delta BR/BPV_I/Neo_I$ has also been introduced into C127 cells by microinjection, analysis of sheared DNAs extracted from eight of the resulting $G418^R$ cell lines and one focus selected cell line was performed (data not shown). None of these cell lines were seen to contain monomeric extrachromosomal forms of the input vector. Again all hybridising material was present as a homogeneous high molecular weight form which migrates near the top of each track.

Thus it would appear that the vector constructs

containing the SV_2Neo transcription unit inserted into pΔBR328/BPV_I do not replicate as an intact, monomeric, extrachromosomal vector in either G418 or focus selected C127 cell lines. This is not an unexpected finding, since it was found (see Chapter 4.5) that the vector pΔBR328/BPV_I itself did not replicate in the desired manner in focus selected transformants generated by the precipitation or injection methods. Apart from the reasons already discussed for pΔBR328/BPV_I, the malfunction of pΔBR328/BPV_I/$Neo_{I \rightarrow IV}$ may be explained by the presence of the SV_2-Neo transcription unit which contains the SV40 viral enhancer sequences. This SV40 enhancer might, in retrospect, compete more efficiently than the BPV-1 enhancer for some cellular factor(s) required by both enhancers, thus repressing expression from BPV-1 sequences. That such effects are possible is suggested by the results of Scholer and Gruss (1984). Alternatively one might speculate that the presence of the SV40 enhancer sequences, in addition to those of BPV-1, might cause overproduction of some finely balanced BPV-1 product, resulting in the aggregation of the vector into a high molecular weight complex. On the other hand Lehn and Sauer (1984) have recently claimed that hybrid genomes containing SV40 early region (enhancer, origin of replication, and SV40 A gene) and the complete BPV-1 genome or the 69% fragment were maintained in an unintegrated state in mouse C127 cells. This suggests that there is no such incompatibility associated with the presence of an SV40 enhancer on a BPV-1

vector. However, little or no plasmid sequences were included in these vectors.

5.7 Analysis of C127 cell lines transformed with pML2d-BPV-1 and pML2d/BPV/MMT-neo

Recently, two vectors, pML2d-BPV-1 (Sarver et al, 1982) and pML2d/BPV/MMT-neo (Law et al, 1983), were obtained from P. Howley (NIH, Bethesda). The vector pML2d-BPV-1 is very similar to $p\Delta AT153/BPV_I$ discussed in chapter 4. It contains the BamHI linear form of BPV-1 inserted into the BamHI site of pML2d, a deletion derivative of pML2 in which the 344bp HindIII/BamHI fragment had been removed and a BamHI linker inserted. This vector was shown by Sarver et al (1982) to replicate as an intact, extrachromosomal monomeric circular form in focus selected C127 cell lines. It was also shown to be recoverable into E.coli upon transformation with low molecular weight DNA and selection for ampicillin resistant colonies. Sarver et al (1982) used the calcium phosphate precipitation method in the presence of carrier DNA to introduce the vector into C127 cells.

Fig. 5.5.a shows an analysis of DNA extracted from seven independently derived C127 cell lines transformed with pML2d-BPV-1 in the absence of carrier DNA under our culture conditions. The DNAs were run as undigested,

FIGURE 5.5.a pML2d/BPV-1 in C127 focus selected cell lines.

Cellular DNA (5µg) was sheared by passage through a 25 gauge syringe. Samples were electrophoresed on a 0.7% agarose gel, transferred to nitrocellulose and hybridised to a nick translated probe, BPV-1. All cell lines were derived by calcium phosphate precipitation.

Samples:-

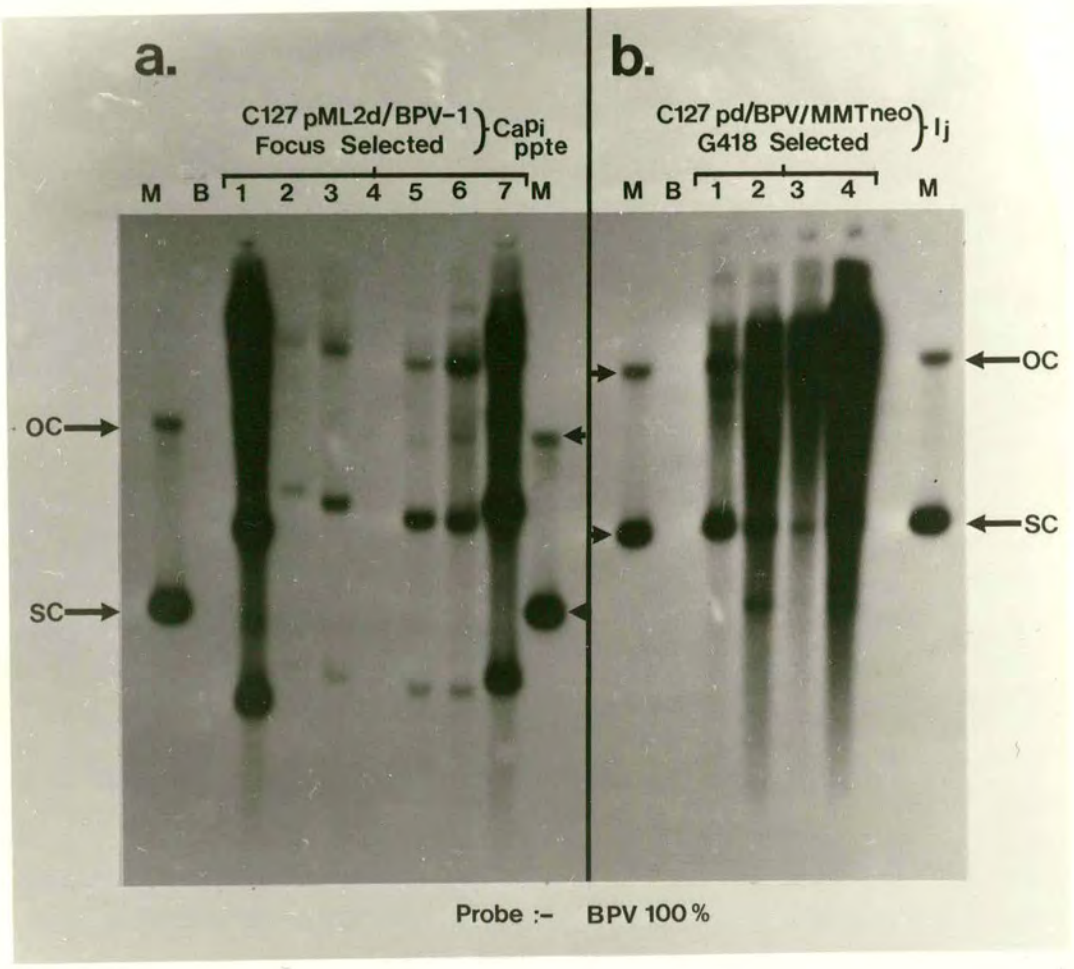
M; 10 copy equivalent of undigested pML2d/BPV-1 input vector in 5µg of sheared Ltap207 DNA. B; 5µg of sheared Ltap207 DNA. 1; DNA extracted from C127 pML2d/BPV-1 Clone 1. 2; Clone 2. 3; Clone 3. 4; Clone 4. 5; Clone 5. 6; Clone 6. 7; Clone 7.

FIGURE 5.5.b pML2d/BPV-1/MMT-NEO in C127 G418 selected cell lines.

Cellular DNA (5µg) was sheared by passage through a 25 gauge syringe. Samples were electrophoresed on a 0.7% agarose gel, transferred to nitrocellulose and hybridised to a nick translated probe, BPV-1. All cell lines were derived by microinjection.

Samples:-

M; 10 copy equivalent of undigested pML2d/BPV-1/MMT-NEO input vector in 5µg of sheared Ltap207 DNA. B; 5µg of sheared Ltap207 DNA. 1; DNA extracted from C127 pML2d/BPV-1/MMT-neo $G418^R$ Ij 204A. 2; Ij 204B. 3; Ij 205A. 4; Ij 205B.


a.
C127 pML2d/BPV-1 Focus Selected
Capi ppte
M B 1 2 3 4 5 6 7 M
OC
SC
b.
C127 pd/BPV/MMTneo G418 Selected
Ij
M B 1 2 3 4 M
OC
SC
Probe :- BPV 100 %

sheared samples and were probed with BPV-1. The autoradiograph shows that in all seven lines no extra-chromosomal forms could be detected which had comigrated with the monomeric circular form of the input plasmid (lanes M). All cell lines contained a smaller extrachromosomal form which is probably between 6-9kb in size (i.e. BPV-1 sized), although this is not visible in lane 4 at this exposure of the autoradiograph. This result shows that under our culture conditions the pML2d-BPV-1 vector did not behave as described by Sarver et al (1982). Each cell line also contained a variable amount of the high molecular weight complex in addition to the deleted extrachromosomal circular forms. No data is yet available for similar cell lines transformed with pML2d-BPV-1 by microinjection.

The vector pML2d/BPV/MMT-neo (Fig 2.11) was constructed by Law et al (1983) by inserting the neo dominant selectable marker attached to the mouse metallothionein (MMT) promoter (MMT-neo) as an EcoRI/BamHI fragment into the pML2d-BPV-1 vector described above. This vector, like $pCGBPV_{9\Delta B5}$ discussed in Section 5.5 was shown to be maintained as an intact extra-chromosomal, monomeric circular form in $G418^R$ C127 cell lines (Law et al, 1983). Because of the results obtained with $pCGBPV_{9\Delta B5}$, the vector pML2d/BPV/MMT-neo was introduced in C127 cells by microinjection only. In Fig. 5.5.b DNA extracted from 4 of the resulting $G418^R$ cell lines is analysed. The DNA was sheared

and the resulting blot probed with BPV-1. As can be seen, the DNA of all four cell lines contained a band which comigrated with the supercoiled form of the input vector (lanes M). However, the cell lines examined in lanes 2 and 4 contained, in addition, smaller circular forms, which presumably represent deleted forms of the input vector in which some BPV-1 and/or plasmid sequences have been deleted. These cell lines also contained a large quantity of the high molecular weight complex. These results again differ from the published data. Unlike $G418^R$ cells transformed by injection with the $pCGBPV_{9\Delta B5}$ vector these $G418^R$ cell lines do contain deleted vector forms.

5.8. Discussion

In this Chapter the behaviour of BPV-1 vectors obtained from other laboratories was investigated in the culture/transformation systems used in this study. All the vectors tested - pBPV-β_1, $pCGBPV_{9\Delta B5}$, pML2d-BPV-1 and pML2d/BPV-1/MMT-neo - had previously been shown to replicate as unrearranged monomeric extra-chromosomal circular forms in transformed C127 cells (DiMaio *et al*, 1982; P. Matthias, pers. comm.; Sarver *et al*, 1982 and Law *et al*, 1983).

Under the culture conditions and cell trans-formation system used in this laboratory, only the pBPV-β_1

vector produced results identical to those of the original workers (DiMaio et al, 1982), when either precipitation or injection methods was employed. The $pCGBPV_{9\Delta B5}$ vector was found to behave as published for $pCGBPV_9$ (Matthias et al, 1983) in $G418^R$ injection-derived C127 cell lines, but most cell lines derived by precipitation contained the vector DNA as a high molecular weight complex. Similar results were found on selection for foci, except that precipitation cell lines contained, in addition, deleted small circular forms. The pML2d-BPV-1 and pML2d-BPV/MMT-neo vectors also gave results which differed from the original results of Sarver et al (1982) and Law et al (1983), respectively.

These results do not shed any light on the discrepancies found in the behaviour of vectors described in Chapters 3 and 4. However, they do suggest that the initial testing of a newly constructed BPV-1 vector should be performed by microinjection rather than precipitation. The former would seem to be more reliable in that if a vector is going to function in the desired manner it will do so if microinjected. Precipitation experiments seem to give highly variable results, which may be a consequence of the culture medium or serum used, or the quality of the DNA precipitate or both. The possibility that the BPV-1 isolate used in the vectors constructed in this thesis is at fault still exists, but this would seem unlikely since the vector pML2d-BPV-1 tested in this Chapter (5.7) and which contains the P.

Howley BPV-1 isolate, and the pΔAT153/BPV_I vectors tested in Chapter 4 are very similar. Both gave rise to very similar results when introduced into C127 cells by the precipitation method (compare Fig. 5.5a with 4.5) suggesting that the structures of the deleted forms in the C127 transformants are produced and selected by the same process.

The results do show that it is possible to use a selectable marker, such as the TK-Neo transcription unit present in $pCGBPV_{9\Delta B5}$, to obtain $G418^R$ transformants which contain a replicating vector as intact monomeric circles. The inclusion of such a dominant selectable marker in the BPV-1 based vector system is an important step since this allows the vector to be introduced into any mammalian cell type free of the BPV-1 focus selection system.

The results obtained in $G418^R$ Ltap207 cells transformed with $pCGBPV_{9\Delta B5}$ by either precipitation or injection were disappointing. They suggest that BPV-1 DNA is not capable of maintaining monomeric extra-chromosomal replication in all mammalian cell lines and that other cell specific factors might be involved. This is perhaps not surprising since BPV-1 virus can only productively infect squamous epithetlial cells in cattle. Therefore, BPV-1 can, in some way, respond to a specific cellular environment. Other attempts to obtain extrachromosomal replication of BPV based vectors in

mouse L cells have failed. These include the pBR328/BPV/TK2 vector (Chapter 3), the pBR328/TK/BPV_I and pΔBR328/TK/BPV_I vectors (Chapter 2, Fig. 2.3) and a pBPV-β_1 vector containing the HSV-TK gene (D.DiMaio, pers. commun.) On the other hand Lusky and Botchan (1984) and Sekiguchi et al (1983) report the extrachromosomal replicaton of BPV-1 containing vectors in L TK^- cells.

Of the vectors reported to be maintained as monomeric circles in C127 transformants, $pCGBPV_{9\Delta B5}$ is possibly the most useful. Not only does it contain a dominant selectable marker, but it also contains both a λ cos site and a single BamHI site thus allowing the insertion of both large and small DNA fragments. The last property that a vector is required to possess is that it should retain its ability to replicate as a monomeric circle even in the presence of a DNA insert (see Chapter 1, property number 7).

In collaboration with J. Dahlberg and E. Lund (University of Wisconsin, Madison, U.S.A.), the human U1 snRNA gene was cloned into the BamHI site of $pCGBPV_{9\Delta B5}$ site in both orientations. These vectors ($pCGBPV_{9\Delta B5}$-HU1-1Do or 1Ds) were microinjected into mouse C127 cells and $G418^R$ transformants selected. On analysis of DNAs extracted from these transformants, it was found that the vector DNA was only present as a high moleular weight complex and no intact small circular forms could

be detected. These results suggest that the human U1 snRNA gene insertion in some way interfered with the extrachromosomal replication of the vector. In this particular case, one possible explanation for the failure to replicate extrachromosomally may be that it is a consequence of physical constraints imposed by the close proximity of genes transcribed by RNA polymerase II (BPV-1$_{\Delta}$ &TK-NEO) and RNA polymerase III (U1 snRNA gene).

Insertion of some other genes into the pBPV-β_1 vector has proved to be successful. Zinn et al (1983) have reported the regulated expression of the human β interferon gene from extrachromosomal forms of the vector. However, when Di Maio et al (1984) inserted a human HLA heavy chain gene into the pBPV-β_1 vector, the resulting C127 cell transformants contained all the vector DNA as a high molecular weight complex and it was concluded that this was integrated into the host genome.

These results suggest that even though a vector may replicate in the desired manner in C127 cells, it may not do so when a gene of interest is inserted into the vector or when a different host cell is utilised. Clearly, such behaviour of these vectors cannot be tolerated if they are to be used in experiments which involve the cloning of random genomic DNA fragments and screening the recombinants in mammalian cells for the expression of an identifiable gene or some other characteristic. The recombinant containing the gene of

interest might integrate and therefore might not be expressed, or, if expressed, would not be easily recoverable into E.coli. Obviously further work on the construction of BPV-1 vectors as 'shuttle' vectors is required. Such work should involve the identification and characterisation of any specific DNA sequences, structural arrangements or other factors that inhibit these vectors from replicating as unrearranged, non-integrating, monomeric extrachromosomal circular forms in mammalian cells. Only when such analyses have been completed might the cloning strategies such as that outlined in the above scenario, be developed with bovine papilloma virus based vectors.

CHAPTER 6

THE RECOVERY OF BPV-1-PLASMID HYBRID DNAs FROM TRANSFORMED MOUSE CELLS INTO BACTERIA

6.1 Introduction

One of the properties required of a BPV-1 shuttle vector is that it should be recoverable back into bacteria after analysis of its replication, expression etc. in a transformed mammalian cell line. Such a property would facilitate the recovery of sequences coding for an identifiable product/function when a library which has been constructed in a BPV-1 vector is subsequently screened following introduction into mammalian cells. A system which allows the shuttling of a vector containing randomly cloned DNA sequences between animal and bacterial cells would therefore be a particularly useful tool in cloning. It would also allow investigations to be carried out on the frequency of mutation in such vectors on passage through mammalian cells for short or long periods. For example, short term experiments by Calos et al (1983) have revealed that transfected DNA suffers a high frequency of mutation in mammalian cells, so the use of a recoverable BPV-1 vector would allow long term studies on mutation frequency to be carried out. A shuttle system of this type requires that the bacterial origin of replication and an antibiotic resistance marker are maintained intact on passage through the mammalian cell.

This Chapter describes attempts which were made to recover the transforming vector DNA from eighteen different cell lines which had been transformed with a

variety of vectors. The particular cell lines were selected on the basis of the presence of an extra-chromosomal band migrating at the same or similar position to that of the supercoiled monomeric input plasmid form when undigested DNAs were analysed by blot hybridisation. The method used for recovery is detailed in Section 2.12 and Fig. 2.2 and ensures that any recovered forms arose from a vector which had undergone replication in a mammalian cell rather than from contaminating bacterial plasmid.

6.2. Analysis of plasmids recovered from BPV-1-plasmid transformants

Out of the eighteen separate recovery experiments attempted, seven cell lines transformed with one of four different vectors yielded bacterial transformants when DNA enriched for circular forms purified from 25μg of total cellular DNA by ExoV treatment was used to transform competent DH1 cells (see Table 6.1.)

6.2.A. Recovery of pBRd-BPV-β_1 amp^R plasmids

In two separate experiments (7A and 7B) the cell line C127 pBRd BPV-β_1 Clone 1 gave ampicillin resistant colonies. However, the experiment A gave 52 ampicillin resistant colonies whereas the second

TABLE 6.1. RESCUE OF REPLICATING VECTOR IN MAMMALIAN CELLS INTO BACTERIAL CELLS

Sample No.	Cell line	Antibiotic Resistance carried by vectors replicating in mammalian cells	Antibiotic Resistance carried by added plasmid prepared from dam^+ bacteria	RESCUED COLONIES: No. of recovered colonies after treatment with ExoV & DpnI Selecting for vector in mammalian cells	CONTROL*: No. of colonies after ExoV treatment only. Selecting for added dam^+ bacterial grown plasmid ~50µg of $pCGBPV_{9\Delta B5}$ ~25µg of pBR328	CONTROL Ø: No. of colonies after ExoV & DpnI treatment selecting for added dam^+ bacterial grown plasmid
#1	C127pCGBPV$_{9\Delta B5}$ G418^R Cl 6	Kan	Amp/Tet/Cm	0	13	0
#2	C127pCGBPV$_{9\Delta B5}$ G418^R 72B†	Kan	"	2	2	0
#3	C127pCGBPV$_{9\Delta B5}$ focus 62A†	Kan	"	0	17	0
#4	C127pCGBPV$_{9\Delta B5}$ focus 65A†	Kan	"	0	6	0
#5	C127pBR328/BPV$_I$/TK dam$^+$ focus 1.1. rc	Amp	Kan	0	6	1
#6	Ltap207 TK$^+$ pBR328/PBV$_I$/TK dam$^-$ Cl.3	Amp	Kan	0	18	1
#7^A	C127 pBrdBPV-β_1 Cl1	Amp	Kan	52	3	0
#7^B	C127 pBrd BPV-β_1 Cl1	Amp	Kan	4	N.D.	N.D.
#8	C127 pΔAT153/BPV$_I$/EN-DUP D_3	Amp	Kan	1	25	0
#9	" " D_4	Amp	Kan	2	4	0
#10	" " C_1	Amp	Kan	0	5	0
#11	" " C_3	Amp	Kan	0	9	0
#12	" " B_1	Amp	Kan	0	11	0
#13	" " B_2	Amp	Kan	2	20	0
#14	" " B_3	Amp	Kan	31	17	0
#15	C127 pΔAT153/BPV$_{II}$ Cl 5	Amp	Kan	0	4	0
#16	C127 pΔIR/BPV$_{II}$ Cl 2	Amp	Kan	0	17	0
#17	C127 pΔBR328/BPV$_I$ 32.B†	Amp	Kan	3	5	0
#18	C127 pΔBR328/BPV$_I$Neo (I→IV) pool G418^R	Amp/CM & Kan	Kan	0	6	0

† - indicates that transformant was obtained by microinjection of the vector DNA.

* - variability indicates the transformation frequency was variabile within a 10-fold 1 x 10^5 → 1 x 10^6 in DH_1.

Ø - indicates that all of the rescued plasmids were not bacterial grown contaminants.

experiment, B gave only 4 (see Table 6.1). Since both DNAs were prepared in the same manner and the same batch of freshly prepared competent cells was used, this 13-fold difference in transformation efficiency must reflect variability either in the quality of the final DNAs or in the actual different transformations. Plasmid DNA was prepared from 6 amp^R colonies derived by transformation with sample 7^A (7^A.1-6) and the 4 amp^R colonies obtained upon transformation with 7^B (7^B.1-4). An aliquot of each undigested preparation was run together with the input plasmid pBRd BPV-β_1 as marker (Fig. 6.1.a). As can be seen all the recovered plasmids were identical in size to the input vector. The identity of these recovered plasmids was further confirmed by comparing the products of BamHI digestion, which should give rise to fragments of size 10.5, 3.5, 1.8 and 0.6kb. All recovered pBRd/BPV-β_1 plasmids (which digested lanes 2,3,4,5,6,7, 9 & 10. Fig. 6.1.b) gave rise to bands of the above sizes, identical to those generated upon BamHI digestion of input vector DNA (Lanes M). The minor bands visible in the digests of recovered bands are partial digestion products.

This result confirms the findings of DiMaio et al (1982) in that the vector pBRd BPV-β_1 can be introduced into mouse C127 at a high efficiency and that the resulting foci contain intact extrachromosomally replicating monomeric forms (see Chapter 5.3 and Fig. 5.1) which are recoverable into bacteria. The vectors

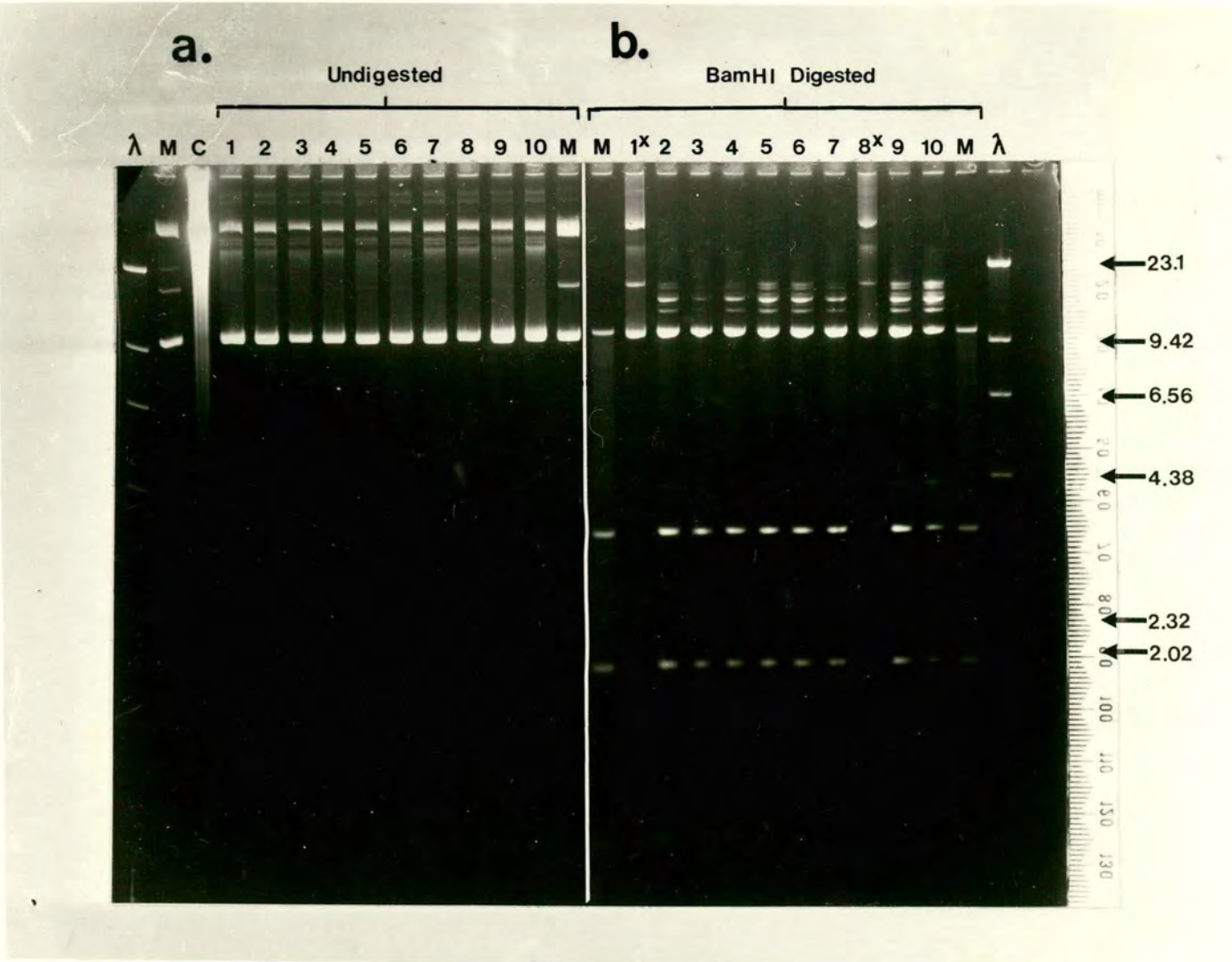
FIGURE 6.1. Agarose gel electrophoresis of plasmid DNA rescued from the cell line C127 pBRd/BPV-β_1 clone 1 (see samples 7^A and 7^B Table.6.1).

Plasmid which had been rescued from this cell line by transfer into E.coli was prepared by the mini-rapid boiling method (Holmes and Quigley, 1981, see Section 2.2.1.B) from a total of 10 of the ampicillin resistant colonies. Samples were analysed undigested (a.) or digested with BamHI (b.), electrophoresed through a 1% agarose gel, which was then stained with ethidium bromide.

Samples:

λ ; HindIII digest of bacteriophage λ DNA (sizes indicated by arrows). M; Input plasmid pBRd/BPV-β_1.
C; Undigested DNA extracted from C127 pBRd/BPV-β_1 Clone 1. 1; Plasmid DNA extracted from amp^R colony 7^A.1. 2; 7^A.2. 3; 7^A.3. 4; 7^A.4. 5; 7^A.5. 6; 7^A.6. 7; 7^B.1. 8; 7^B.2. 9; 7^B.3. 10; 7^B.4.

x : indicates that sample did not digest with BamHI.


a.
Undigested
λ M C 1 2 3 4 5 6 7 8 9 10 M
b.
BamHI Digested
M 1x 2 3 4 5 6 7 8x 9 10 M λ
23.1
9.42
6.56
4.38
2.32
2.02

have thus retained both the plasmid replication origin and the ampicillin resistance marker.

6.2.B. Recovery of $pCGBPV_{9\Delta B5}kan^R$ plasmids

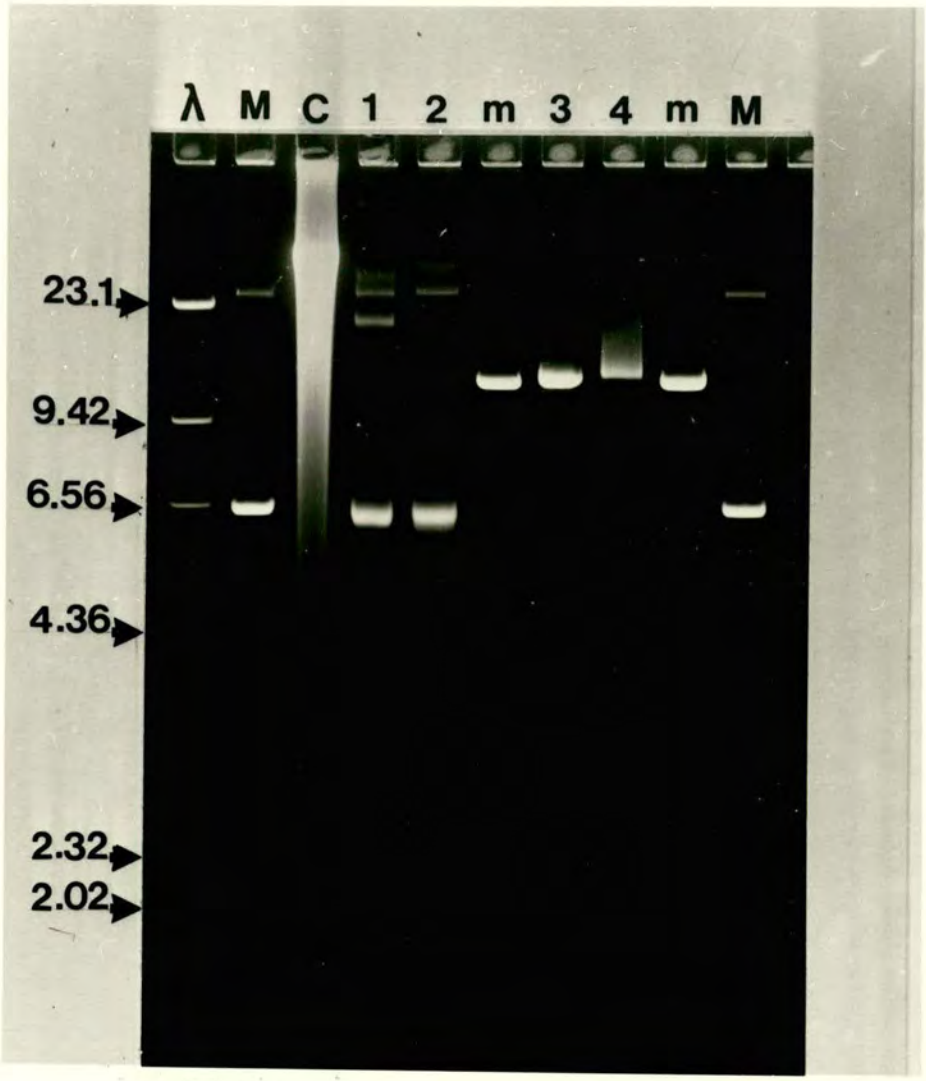
In Section 5.5. (Fig. 5.2A, lanes 7-14) it was demonstrated that the vector $pCGBPV_{9\Delta B5}$, which carries the TK-NEO $G418^R$ selectable marker, replicated efficiently as an unrearranged extrachromosomal monomeric circular plasmid in G418 selected C127 transformants derived by microinjection. Two cell lines arising by focus selection after injection were also thought to contain intact vector (Fig. 5.2B, Lane 10 and 15). A small proportion of intact episomal $pCGBPV_{9\Delta B5}$ vector was also seen in one $G418^R$ cell line derived by the precipitation method (Fig. 5.2.A, lane 5). Attempts to recover plasmid from 25µg of these ExoV/DpnI treated total cellular DNAs resulted in only two kan^R colonies. These were obtained using DNA from the $G418^R$ injected cell line C127 $pCGBPV_{9\Delta B5}Ij$ 72B (sample #2 Table 6.1). Examination of these recovered plasmids revealed that they were identical in size to both undigested input vector (Fig. 6.2, lanes M, 1 and 2) and BamHI digested input vector (Fig. 6.2., lanes m, 3 and 4). This result confirms those of Matthias et al (1983) in that it demonstrates that the monomeric replicating forms of $pCGBPV_{9\Delta B5}$ in $G418^R$ C127 cell lines can be recovered, intact, into E.coli. The

FIGURE 6.2. Agarose gel electrophoresis of plasmid DNA rescued from the cell line C127 $pCGBPV_{9\Delta B5}$ $G418^R$ Ij 72B (see sample 2 Table.6.1).

Plasmid which had been rescued from this cell line by transfer into E.coli was prepared by the mini-rapid boiling method (Holmes and Quigley, 1981, see Section 2.2.1.B.) from both of the kanamycin resistant colonies. Samples were analysed undigested (lanes 1 & 2.) or digested with BamHI (lanes 3 & 4), electrophoresed through a 1% agarose gel, which was then stained with ethidium bromide.

Samples:

λ ; HindIII digest of bacteriophage λ DNA (sizes indicated by arrows). M; Undigested input plasmid $pCGBPV_{9\Delta B5}$. m; BamHI digested input plasmid $pCGBPV_{9\Delta B5}$. C; Undigested DNA extracted from C127 $pCGBPV_{9\Delta B5}$ Ij 72B. 1; Plasmid DNA extracted from kan^R colony 2.1 undigested. 2; 2.2 undigested. 3; 2.1 BamHI digested. 4; 2.2 BamHI digested.


λ M C 1 2 m 3 4 m M
23.1
9.42
6.56
4.36
2.32
2.02

fact that no kan^R colonies were obtained with samples Nos. 1, 3 or 4 (Table 6.1) suggests that any intact replicating forms were present at levels below that which would have enabled them to be recovered in the individual transformations.

6.2.C. Recovery of pΔAT153/BPV_I/EN-DUP plasmids

In Chapter 4 the construction of the vectors pΔAT153/BPV_I/EN-DUP B, C, and D was described. Out of a total of 16 cell line DNAs examined (5 x pD, 6 x pC and 5 x pB transformants), 7 were found to contain a band which comigrated with the supercoiled monomeric circular form of the input vector (Fig.4.7). Attempts were made to recover these putative intact replicating forms into E.coli from these seven cell lines (Table 6.1. samples #8-#14). Again, 25 µg of ExoV/DpnI treated DNA were used to transform competent DH_1 cells, which were then selected for resistance to ampicillin. Four of these cellular DNAs (D3, D4, B2 and B3) gave rise to ampicillin resistant colonies (samples #8, #9, #13 and #14, respectively, in Table 6.1). Examination of plasmid extracted from these colonies revealed that all contained a supercoiled and open circular form of a plasmid which comigrated with that of the input vector pΔAT/153BPV_I/EN-DUP D or B (pEN-$DUPD_3$ 8.1 undigested Fig. 6.3.A, lane 1, pEN-$DUPD_4$ 9.1 and 9.2 undigested Fig. 6.3.B. lane 1 and 2, pEN-$DUPB_2$ 13.1 and 13.2 undigested

FIGURE 6.3. Agarose gel electrophoresis of plasmid DNA rescued from the cell lines (A) C127 pΔAT153/BPV$_I$/EN-DUP-D.3 (see sample 8 Table 6.1). (B) C127 pΔAT153/BPV$_I$/EN-DUP-D.4 (see sample 9 Table 6.1). (C) C127 pΔAT153/BPV$_I$/EN-DUP-B.2 (see sample 13 Table 6.1).

Plasmid which had been rescued from these cell lines by transfer into E.coli was prepared by the mini-rapid boiling method (Holmes and Quigley, 1981, see Section 2.2.1.B) from these ampicillin resistant colonies. Samples were analysed undigested (lanes A.1, B.1, B.2, C.1 & C.2) or digested with BamHI (lanes A.2, B.3 & B.4), electrophoresed through a 1% agarose gel, which was then stained with ethidium bromide.

Samples:

λ ; HindIII digest of bacteriophage λ DNA (sizes indicated by arrows). M; Undigested input sized plasmid pΔAT153/BPV$_I$/EN-DUP-D. m; BamHI digested input sized plasmid pΔAT153/BPV$_I$/EN-DUP-D. C; Undigested DNA extracted from the various starting cell C127 lines in A, B & C. A.1; Plasmid DNA extracted from amp^R colony 8.1 undigested. A.2; 8.1 BamHI digested. B.1; 9.1 undigested. B.2; 9.2 undigested. B.3; 9.1 BamHI digested. B.4; 9.2 BamHI digested. C.1; 13.1 undigested. C.2; 13.2 undigested.

x : indicates that sample did not digest with BamHI.

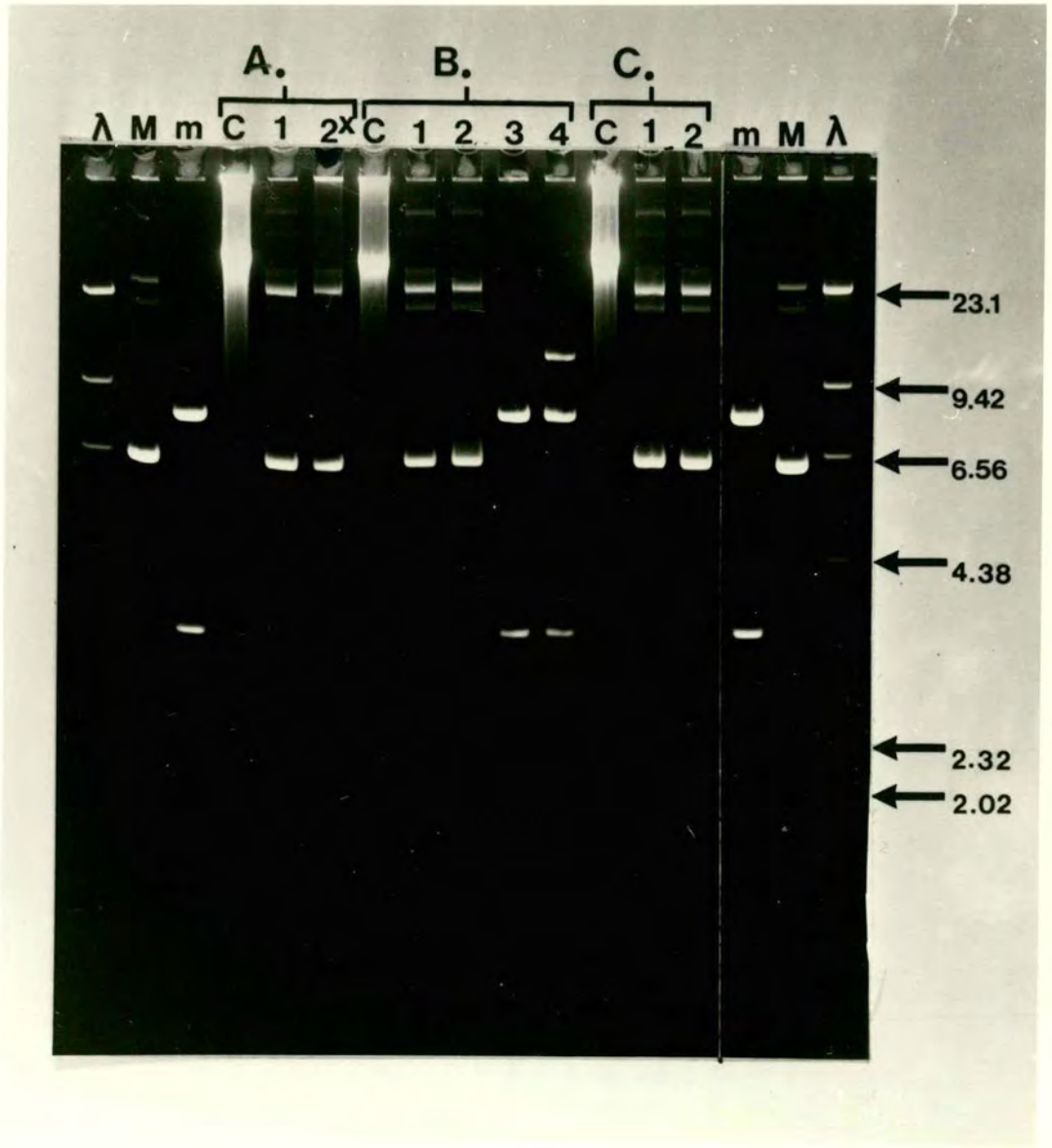

A.
B.
C.
λ M m C 1 2x C 1 2 3 4 C 1 2 m M λ
23.1
9.42
6.56
4.38
2.32
2.02

FIGURE 6.4. Agarose gel electrophoresis of plasmid DNA rescued from the cell line C127 $p\Delta AT153/BPV_I/EN\text{-}DUP\text{-}B.3$ (see sample 14 Table.6.1).

Plasmid which had been rescued from this cell line by transfer into E.coli was prepared by the mini-rapid boiling method (Holmes and Quigley, 1981) from a total of 10 of the ampicillin resistant colonies. Samples were analysed undigested (a.) or digested with BamHI (b.), electrophoresed through a 1% agarose gel, which was then stained with ethidium bromide.

Samples:

λ ; HindIII digest of bacteriophage λ DNA (sizes indicated by arrows). M^*; Undigested input sized plasmid $p\Delta AT153/BPV_I/EN\text{-}DUP\text{-}D$. C; Undigested DNA extracted from C127. $p^{\Delta}AT153/BPV_I/EN\text{-}DUP\text{-}B.3$. 1; Plasmid DNA extracted from amp^R colony 14.1. 2; 14.2. 3; 14.3. 4; 14.4. 5; 14.5. 6; 14.6. 7; 14.7. 8; 14.8. 9; 14.9. 10; 14.10.

x : indicates that sample did not digest with BamHI.

: in BamHI digests of the input plasmid some $pCGBPV_{9\Delta B5}$ was also included (11.3Kb linear)

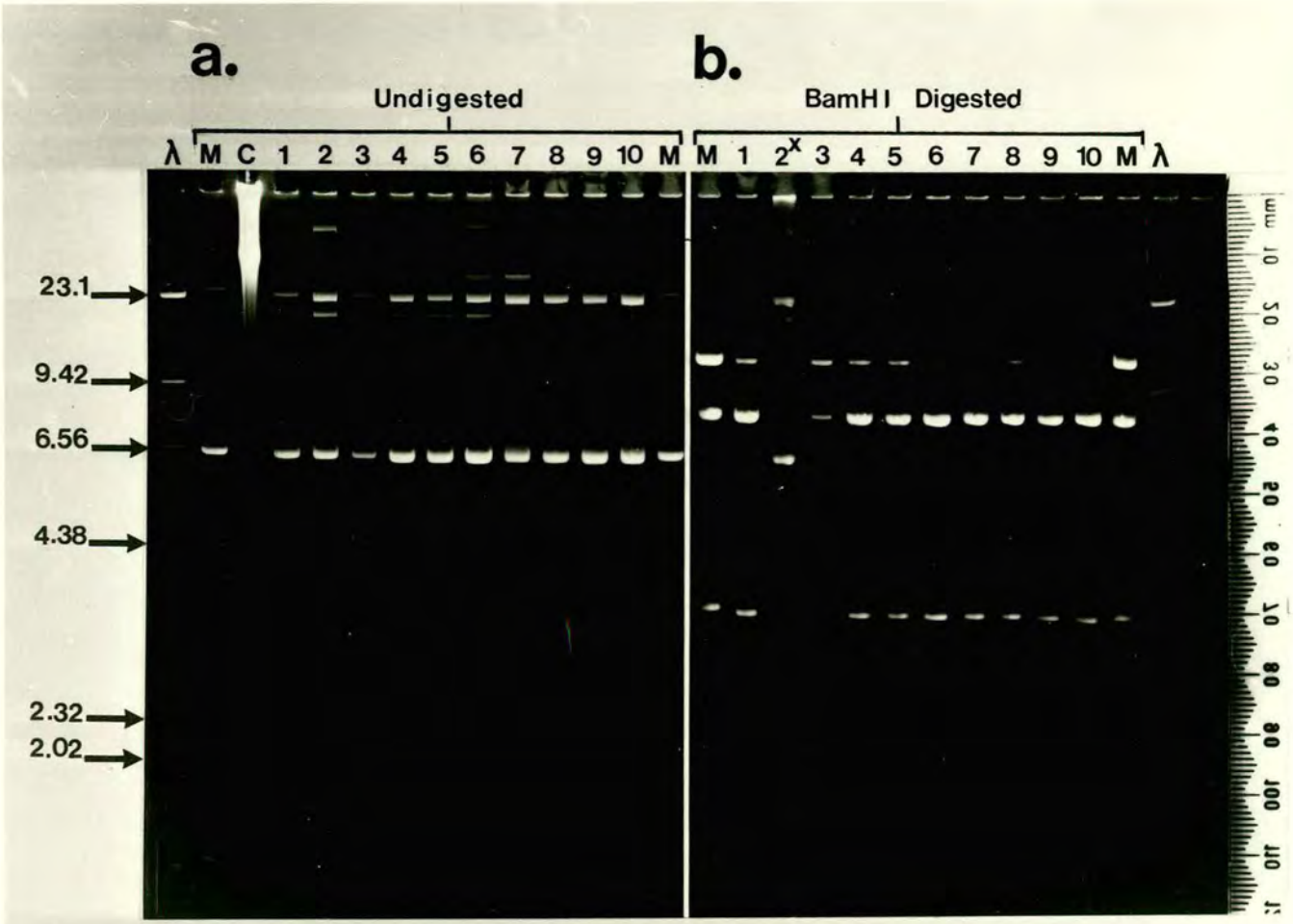

a.
b.
Undigested
BamHI Digested
λ M C 1 2 3 4 5 6 7 8 9 10 M
M 1 2x 3 4 5 6 7 8 9 10 M λ
23.1
9.42
6.56
4.38
2.32
2.02

Fig. 6.3.C. lane 1 and 2, pEN-DUP B_4 14.1-14.10. Fig. 6.4.a, lane 1-10). Further examination of these recovered plasmids by BamHI digestion, which releases the BPV-1 from pΔAT153, resulted in the expected band sizes of 8.0kb (BPV-1) and 3.3kb (pΔAT153) (see Fig. 6.3.A, lane 2, Fig. 6.3.B, lane 3 and 4, Fig. 6.3.b lanes 1-10). This result shows that 4/7 of those cell lines, which were seen in Fig. 4.7 to contain a band corresponding to the monomeric supercoiled input form of the vector, do indeed contain intact vector DNA which has replicated in the C127 cells and is recoverable into bacteria.

It is of interest to note that none of the pEN- DUP C (sample numbers 10 and 11, table 1) transformed cell lines gave rise to any recoverable vector. Of further interest is the fact that samples #15 and #16, which were transformed with pΔAT153/BPV_{II} and pΔIR/BPV_{II}, respectively, and which contained a large quantity of material which comigrated with or close to the super-coiled monomer of the input vectors (Fig. 4.4., lane 2 and Fig. 4.5, lane 11 respectively) did not give rise to ampicillin resistance colonies when transformed into DH_1. It is possible that this resulted from a qualitative difference in the individual transformations due either to the DNAs or the E.coli cells, but in each case the internal control gave rise to kan^R colonies. It is also possible that recoverable forms were present in the DNA samples but at a level below that which would have been detected by the transformation system.

These results confirm that the pEN-DUP B & D vectors do replicate as recoverable intact extrachromosomal forms in transformed mouse C127 cell lines, and suggest that duplication of the BPV-1 enhancer in these vectors does in some way create a more stable form, especially when compared to pΔAT153/BPV$_{I \text{ or } II}$ analysed in Fig. 4.5. It should be noted that the C127 pΔAT153/BPV$_I$/ EN-DUP cell lines from which amp^R colonies were recovered were those in which relatively few smaller deleted extrachromosomal forms of the vector were detected (see Fig. 4.7, lanes, 3, 4, 13 and 14).

6.2.D. Recovery of deleted forms of the pΔBR328/BPV$_I$ vector

One cell line of interest, from which an attempt was made to recover replicating forms, was a C127 cell line which had been transformed with pΔBR328/BPV$_I$ by microinjection (C127 pΔBR328/BPV$_I$,I$_j$ 32B, sample #17, Table 6.1). This cell line was chosen for recovery because of the unusual pattern of extrachromosomal bands seen in this one transformant (see Section 4.5). It was possible that it contained some intact input vector DNA.

Upon transformation of E.coli DH1, 3 ampicillin resistant colonies were obtained (labelled 17.1, 17.4 and 17.5). Analysis of plasmid DNA extracted from these,

either undigested (lanes 1-3) or digested with BamHI (lanes 4-6), is shown in Fig. 6.5. Rather than being identical to the input vector DNA, these plasmids are smaller than the input vector. Only one plasmid, 17.4, was digestable with BamHI (Fig. 6.5, lane 5), which revealed that the linear form of the plasmid migrated at approximately 4.2kb. It must therefore have lost approximately 7.4kb of the original vector sequence. Further attempts to digest 17.1 and 17.5 revealed that 17.1 is probably identical to 17.4. However, 17.5 is only about 3kb in size (data not shown).

These deleted forms were probably present in the original cellular DNA because extrachromosomal forms of these sizes were detected by hybridisation with pBR328/BPV$_I$ (data not shown). Probing the recovered plasmids 17.4 and 17.5 with BPV-1, however, revealed the presence of viral sequences only in 17.4, and no hybridisation to 17.5 could be detected (data not shown). This suggests that, either 17.5 was a contaminant of some sort, that BPVI had been completely deleted or that the amount of BPV-1 sequence remaining in the vector was so small as to remain undetectable by hybridisation with BPV-1 alone. Further mapping of these deleted forms of pΔBR328/BPV-1 is necessary to establish which BPV-1 sequences, if any, they contain and whether they could form a useful basis for a BPV-1 mini-vector.

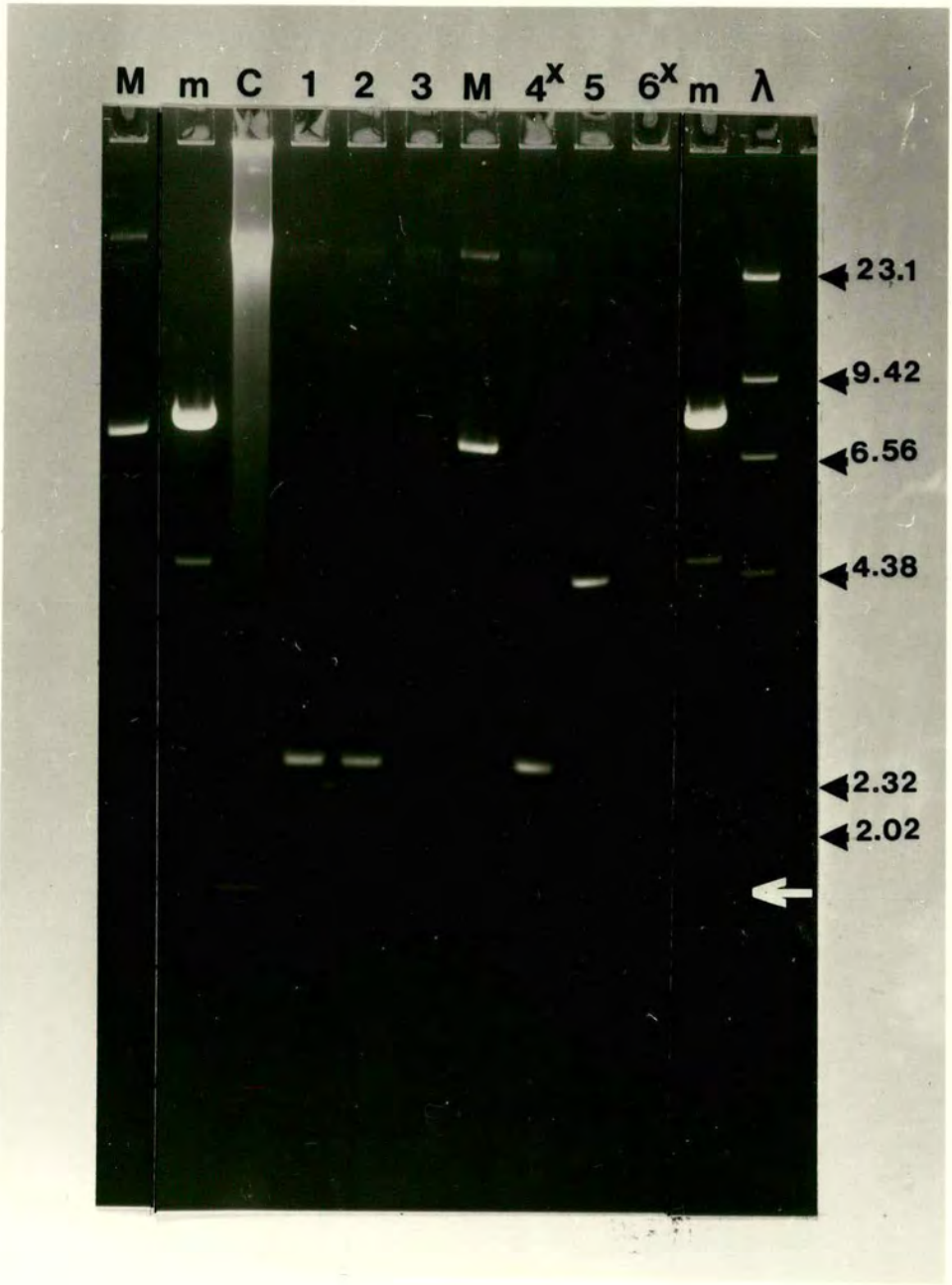
FIGURE 6.5. Agarose gel electrophoresis of plasmid DNA rescued from the cell line C127 $p\Delta BR328/BPV_I$ Ij 32B (see sample 17 Table.6.1).

Plasmid which had been rescued from this cell line by transfer into E.coli was prepared by the mini-rapid boiling method (Holmes and Quigley, 1981, see Section 2.2.1.B) from the three ampicillin resistant colonies obtained. Samples were analysed undigested (lanes 1, 2, & 3) or digested with BamHI (lanes 4, 5, & 6), electrophoresed through a 1% agarose gel, which was then stained with ethidium bromide.

Samples:

λ ; HindIII digest of bacteriophage λ DNA (sizes indicated by arrows). M; Undigested input sized plasmid $p\Delta BR328/BPV_I$.
m; Products of BamHI digestion of $p\Delta BR328/BPV_I$
C; Undigested DNA extracted from C127. $p\Delta BR328/BPV_I$
Ij 32B. 1; Plasmid DNA extracted from amp^R colony
17.1 undigested. 2; 17.4 undigested. 3; 17.5 undigested.
4^x; 17.1 BamHI digested. 5; 17.4 BamHI digested.
6^x; 17.5 BamHI digested.

x : indicates that sample did not digest with BamHI.
White arrow : indicates position of supercoiled form of plasmid 17.5 in lane 3, which is very faint.


M
m
C
1
2
3
M
4x
5
6x
m
λ
23.1
9.42
6.56
4.38
2.32
2.02

6.3. Discussion

The ability to recover intact plasmids from the DNA of cells transformed with the pEN-DUP B & D vectors suggests that the duplication of the viral enhancer in the opposite orientation to the endogenous enhancer either directly adjacent to it (pEN-DUP/B) or at the opposite end of the BPV-1 insert (pEN-DUP/D) has some effect which increases the stability of the vector in C127 cells. Whether or not the vectors had undergone some minute favourable rearrangement following entry into the cell remains to be seen. It is possible that a small change in its structure would be undetectable by restriction enzyme digestion, but the ability of these recovered vector forms to replicate consistently as unrearranged forms in C127 cells during a second round of transformation is currently being investigated.

Likewise the deleted forms of the pΔBR328/BPV_I vector, 17.4 and 17.5, recovered as ampicillin resistant plasmids are being further investigated by secondary transformation of C127 cells. The possibility exists that these forms of the vector contain the minimal BPV-1 sequences required for replication. For example, they may have retained a BPV-1 PMS element, shown by Lusky and Botchan (1984) and Waldeck *et al* (1984) to be necessary for replication. Perhaps these deleted forms will only replicate in C127 provided the correct viral functions are provided in *trans* by an endogenous

intact BPV-1 genome. This hypothesis is currently being investigated and may provide further insight into the requirements for the replication of BPV-1 in transformed fibroblasts.

It has therefore been demonstrated here and elsewhere (DiMaio et al, 1982; Sarver et al, 1982, Matthias et al, 1983) that some of the vectors examined can be shuttled efficiently between transformed mouse cells and competent bacteria. This property suggests that these vectors may be useful for introducing libraries of heterogeneous DNAs cloned in these vectors into mammalian cells and subsequently may allow the selection and rapid isolation of the DNA sequence encoding a particular phenotypic function. Further work on these vectors is required to investigate the inhibitory effects that some cloned genes impart on the stability of these vectors when reintroduced into mammalian cells.

CHAPTER 7

THE STRUCTURE OF BPV-1 DNA IN A TRANSFORMED MOUSE CELL LINE

7.1. Introduction

The data presented in the preceeding Chapters have shown that in cells transformed by BPV-1, or BPV-1-containing vectors, the resulting cell lines were seen to contain circular DNA forms. In most cases the smallest forms represented deleted or intact supercoiled monomeric molecules resulting from the replication of input DNA. In addition many cells also contained a series of bands which migrated at higher positions in the gel track and which eventually merged into, or became masked by, a large heterogeneous smear present at the position of high molecular weight linear DNA (i.e. >30kb). In this Chapter the structural form of these discrete circular bands and the high molecular weight complex are investigated.

In Chapter 3.2 an analysis was carried out on DNA extracted from C127 cell lines transformed by BamHI cleaved pΔBR328/BPV$_I$. The results showed that the BPV-1 isolate ultilised in the construction of all vectors in this thesis behaved in an identical manner to that presented by Law et al (1981). All cell lines were seen to contain monomeric, open and supercoiled circular forms and discrete sized bands could be detected in sheared DNA samples (see Fig. 3.1.A.) migrating more slowly than the monomeric BPV-1 circular forms. In addition, all cell lines contained a large quantity of high molecular weight material. Law et al (1981)

suggested that the high molecular weight complex represented a complex of catenated interlocked monomeric circular DNAs on the basis of conversion of the high molecular weight complex to monomeric open circular and linear forms upon limited S1 digestion. However, this data was not shown. It was also suggested that these forms may represent viral replication intermediates.

The cell lines constructed by Law et al (1981) resulted from the introduction of BamHI cleaved pBR322/BPV-1 vector DNA in the presence of carrier DNA by the calcium phosphate precipitation method. No attempt was made to detect any pBR322 sequences which might have been cotransferred with the BPV-1 BamHI linear DNA into these C127 cells. In Chapter 3.2, probing of pΔBR328/BPV-1/BamHI cleaved transformed cell line DNAs with pBR328 on its own revealed the presence of plasmid sequences in many of the cell lines (Fig. 3.1.D). Significantly, no plasmid sequences were detected in discrete bands in these sheared DNA samples, but were only found in the high molecular weight smear. Probing of BamHI and HindIII digests with pBR328 showed fragments of predictable sizes, suggesting that this high molecular weight complex contained forms which resulted from the relinkage of intact or rearranged pΔBR328 to BPV-1 molecules. It is conceivable that the high molecular weight complex results from the inclusion of plasmid DNA in the transformation mix, but Law et al (1981) have found similar forms in C127 cell lines transformed with

BPV-1 virions, and such a complex can be seen in both a BPV-1 induced bovine fibroma (Lancaster et al, 1981) and hamster cell lines derived from BPV-1 induced tumours (Breitburd et al, 1981). Taken together, these results suggest that such high molecular weight forms are a normal product of BPV-1 replication in bovine, hamster or mouse C127 cells and are not the result of an interaction with linked plasmid sequences. The fact that three cell lines examined (Fig. 3.1, lanes 3, 4 and 13) did contain this high molecular weight complex, but did not contain any hybridising plasmid sequences, provides further evidence that the complex is formed in the absence of plasmid sequences.

To further characterise the nature of this high molecular weight complex as well as the extrachromosomal circular forms the cell line C127 pΔBR328/BPV_I/BamHI C1.2 (lanes 2 of Fig. 3.1, A, B, C, D, E and F,) was used for the extensive analysis presented below.

7.2. Caesium Chloride/Ethidium Bromide Gradient Fractionation of C127 pΔBR328/BPV_I/ BamHI C1.2 total cellular DNA

In order to ensure that the total cell population in the C127 pΔBR328/BPV/BamHI C1.2 cell line was derived from a single cell transformant, and to enrich for transformed cells, the cell line was recloned (rc). This

transformed clone was then grown up in the dark (to prevent nicking) to a semi-confluent state, the cells were lysed, treated with pronase and the total lysate loaded onto a 4.4 ml CsCl/EtBr gradient. After the appropriate centrifugation time the gradient was fractionated from the bottom into 18 x 250µl fractions. Approximately 40% of the DNA recovered from each fraction was electrophoresed on a 0.6% agarose gel, which was then blotted and hybridised with BPV-1 DNA as a probe. The autoradiograph in Fig. 7.1 shows that the supercoil DNA is contained around fraction #2, whereas open circular/ linear fractions centre on fraction #10. Lane P contained a sample removed from the gradient before centrifugation and thus controls for any nicks that may have been introduced into the DNA by the gradient fractionation and recovery procedures. Lanes M contain a sample of total cellular DNA from the original cell line (as seen in Fig. 3.1A). In the supercoiled fractions approximately 15 specific bands can be detected, but some of them probably represent open circular and linear forms caused by nicking during fractionation of the gradient since they comigrated with bands seen at the same position in the open circular/linear fractions.

The open circular fraction is seen to contain a very large quantity of material migrating as a high molecular weight smear, but a small amount of this material is also visible in the supercoil fraction as a diffuse band. This material in the open circular

FIGURE 7.1. Fractionation of a cell lysate from C127 pΔBR328/BPV$_I$/BamHI Clone 2 rc on a caesium chloride/ethidium bromide gradient.

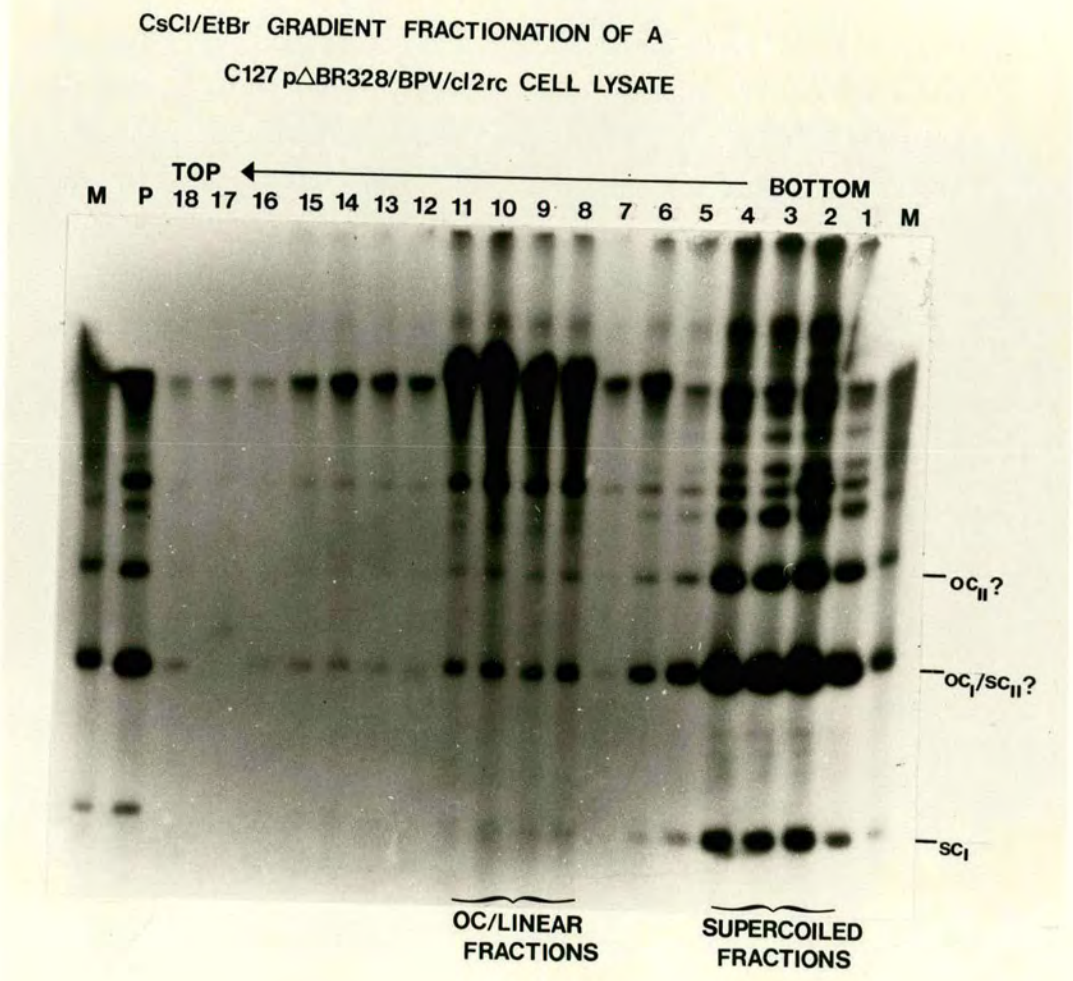
The lysate of approx. 5x10^5 cells was loaded onto a 4.4 ml CsCl/EtBr gradient. After centrifugation the gradient was fractionated from bottom to top into 18 x 250µl fractions. The EtBr and CsCl was removed, an aliquot (2/5) of each fraction was electrophoresed through a 0.6% agarose gel, transferred to nitrocellulose and hybridised to nick translated pBR328/BPV-1/TK$_2$ as probe.

Fractions$^{\#}$1-18 (bottom to top)are as indicated. The fractions which contain open circular &/or linear or supercoiled forms are indicated.

M; Total cellular DNA extracted from the original C127 pΔBR328/BPV$_I$/BamHI Clone 2 cell line.

P; 'Pre'-Sample removed from gradient before centrifugation.

SC$_I$: indicates the position of the monomeric BPV-1 supercoiled circular form. The possible position of other forms is also indicated.


CsCl/EtBr GRADIENT FRACTIONATION OF A
C127 pΔBR328/BPV/cl2rc CELL LYSATE
TOP
BOTTOM
M P 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 M
OC/LINEAR FRACTIONS
SUPERCOILED FRACTIONS
OC_II?
OC_I/SC_II?
SC_I

fraction could either represent chromosomally integrated BPV-1 sequences, broken large circular forms or a large episomal linear structure formed during BPV-1 DNA replication. The diffuse high molecular weight material seen in the supercoiled fraction probably results from the breakage of large circular forms during the treatment of the gradient fractions to remove EtBr etc. To investigate further the many forms seen in the supercoil fraction, samples from fraction #2 have been run on 2-dimensional agarose gels.

7.3. 2-D gel electrophoresis of C127 pΔBR328/BPV$_I$/ BamHI/C1.2rc supercoiled fraction

When a supercoiled form of a circular molecule is briefly irradiated with UV light single stranded nicks are introduced into the molecule, which result in its conversion to the open circular (relaxed) form. Further irradiation will result in nicking which is sufficiently frequent to introduce a double stranded nick. This results in the production of the linear form of the molecule. Because the supercoiled form of a circular molecule migrates faster on agarose gels than do the linear or the open circular forms, the above type of nicking experiment can be followed by running samples of the nicked DNA on agarose gels. If one has a complex mixture of monomeric and oligomeric open circular, linear and supercoil forms, the respective supercoil and open

circular monomers, dimer, etc. can be identified by running 2-dimensional agarose gels (see Figure 7.2). In the first dimension a sample of the DNA mixture is run without any treatment of the DNA. The gel track is then cut out and briefly treated with UV and run in the second dimension which is at right angles to the first. After running in the second dimension any forms which were unaffected by the UV irradiation will have migrated to the diagonal across the centre of the gel. If a supercoil (sc) was nicked by the UV it will have formed an open circular (oc) form, which, in the second dimension will migrate slower than any remaining supercoil and at the same position as any open circular form of the same size that may have been present in the first dimension and which therefore would remain on the diagonal. The relationship of sc_n to oc_n (n represents monomeric to oligomeric forms i.e. I, II, III etc.) can be seen by the presence of a triangular relationship (see Fig. 7.2).

Samples of supercoiled fraction #2 described in the previous section, were loaded onto a 0.6% agarose gel which was run in the first dimension. Two lanes were then removed and treated with UV (i.e. sunlight for 1hr or 7 minutes with a Hanovia Bacteriocidal UV unit - $300\mu Wcm^{-2}$) and each was then placed horizontally across the top of a second 0.6% agarose gel which, after electrophoresis, formed the second dimension. The resulting gels were blotted to nitrocellulose and probed

FIGURE 7.2. Diagram demonstrating the use of two dimensional agarose gel electrophoresis for relating the the open circular, linear and supercoiled circular oligomeric forms of a DNA molecule.

First and second dimensions are as indicated.

SC_I : Monomeric supercoiled circular form.

L_I : Monomeric linear form.

OC_I : Monomeric open circular form.

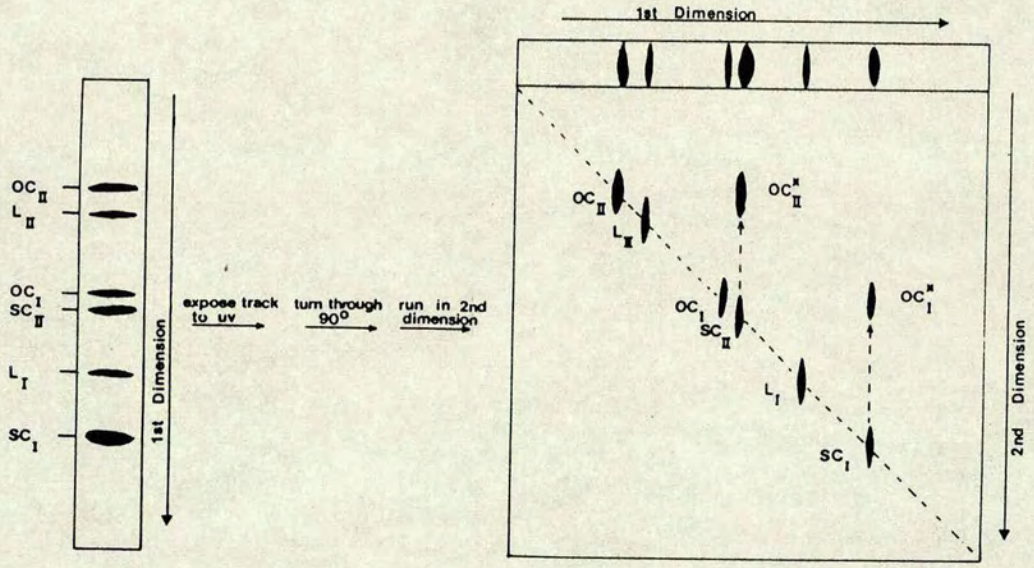
SC_{II}: Dimeric supercoiled circular form.

L_{II} : Dimeric linear form.

OC_{II}: Dimeric open circular form.

OC_I^* & OC_{II}^* : Monomeric and dimeric open circular forms created upon UV irradiation of SC_I and SC_{II} respectively, in the 1st D gel, now comigrate with OC_I and OC_{II} respectively, in the second dimension.

Note: Forms unaffected by UV irradiation remain on the diagonal in the 2nd dimension.


OC II
L II
OC I
SC II
L I
SC I
1st Dimension
expose track to uv
turn through 90°
run in 2nd dimension
1st Dimension
OC II
L II
OC* II
OC I
SC II
OC* I
L I
SC I
2nd Dimension

with BPV-1 DNA. The resulting autoradiograph in Fig. 7.3.A (resulting from sunlight irradiation) clearly shows the triangular relationship between certain bands, which allows the identification of the various forms present in the supercoiled fraction from the CsCl/EtBr gradient.

A schematic interpretation of the different bands seen in Fig 7.3.A (and a lower exposure which is not shown) is presented in Figure 7.3.B. This diagram suggests that all the BPV-1 circular forms present in the C127 pΔBR328/BPV_I/BamHI Cl.2 rc cells were concatomers (oligomeric circles, not interlocked) of the monomeric circular form, and clearly multimers which contained up to six BPV-1 genomes (i.e. sc VI and oc VI) could be identified (48kb circles). The presence of unidentifiable bands in the top left hand corner of Fig.7.3A suggests that these multimers may go up to decamers (80kb) or even larger.

The diagonal shows the presence of linear forms in this fraction, since the mobility of a band which ran at approximately 16kb in the first dimension was uneffected by the UV treatment. This band probably represents a linear dimer form of the 7.95kb BPV-1 genome. The presence of larger linear forms at ~30kb also indicated (Fig. 7.3.B) may result from the random breakage of the large circular forms thus producing the heterogeneous smear seen at this position. (These linears are further investigated in the next Section.)

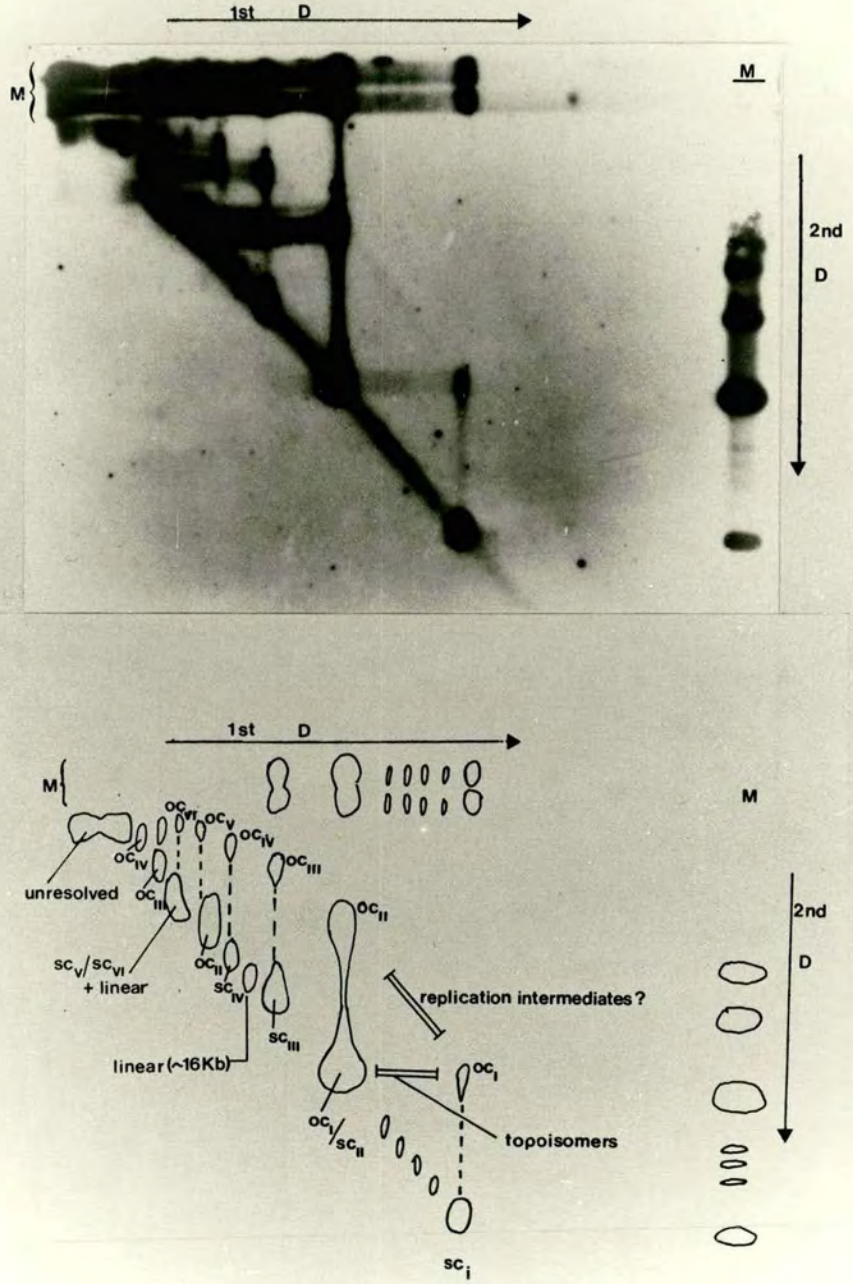
FIGURE 7.3. Two dimensional agarose gel electrophoresis of the supercoiled fraction isolated from the C127 pΔBR328/BPV$_I$/BamHI Clone 2 rc cell line (see FIGURE 7.1).

DNA isolated from the supercoiled fraction of the CsCl/EtBr gradient (see Figure 7.1) was electrophoresed through a 0.6% agarose gel (top, horizontal lanes M, as in Figure 7.1 supercoiled fractions). This first dimension of the gel was left submerged in electrophoresis buffer, in sunlight (UV) for approx. 1 hour. Second dimension electrophoresis through a 0.6% agarose gel was then performed with marker (M : supercoiled fraction from Figure 7.1). The DNA in this two dimensional gel was transferred to nitrocellulose and hybridised with a nick translated probe, pBR328/BPV-1/TK$_2$.

Top Panel (A) : shows the resulting autoradiograph.
Bottom Panel (B) : Shows a schematic interpretation of the results in A.

Note : FIGURE 7.2. diagrammatically demonstrates the use of such gels for relating open circular and supercoiled circular forms.

2-D AGAROSE GEL ELECTROPHORESIS OF C127pΔBR328/BPV/BamH1cl2rc
SUPERCOILED FRACTION



When the gel track treated under a UV lamp for 7 mins was run in the second dimension and analysed by blot hybridisation to BPV-1 DNA, many of the bands seen in Fig. 7.3.A which represented open circular forms gave rise to a band migrating faster which was presumed to represent the linear form (data not shown). In no case were the higher molecular weight multimers (i.e. $>sc_{II}$) seen to give rise to any form migrating with the monomeric linear form. Since most of the monomeric circular forms appeared to have been converted to the monomeric linear form, this result suggests that no catenated forms (interlocked circular monomeric forms) were present in the high molecular weight bands as has been suggested by Law et al (1981).

In Figure 7.3.A some minor forms are labelled as topoisomers and replicative intermediates. These topoisomers may represent monomeric circular forms which have fewer super helical turns than the fully supercoiled monomeric BPV-1 molecules. Upon nicking however, these forms were converted to the monomeric open circular forms. The BPV-1 genome is known to replicate as a 'Cairns' type structure (Waldeck et al, 1984). The 'Cairns' type replication intermediates contain one replication eye in replicating molecules. Therefore, if BPV-1 replication was in some way synchronous, it is possible that specific bands would be seen in cellular DNA which would migrate at a position slower than the

monomeric unreplicating BPV-1 supercoil but faster than a complete supercoil dimer. Upon nicking with UV, bands were detected which migrated at positions intermediate between the monomeric and dimeric open circular forms, which is consistent with them being 'Cairns' type replication intermediates.

This result suggests that BPV-1 DNA can be present as supercoiled concatemeric forms in BPV-1 transformed cell lines. These concatemeric circles may contain up to 10 or more BPV-1 genomes resulting in circular forms of up to 80kb in these cells. Whether these large circular forms are unresolvable products of BPV-1 replication or whether they represent BPV-1 multimers which would eventually be packaged into viral particles in a productive infection is not known. In this experiment, in mouse C127 cells, BPV-1 replication does not appear to give rise to catenated dimers (or multimers) as has been found to be the case with SV40 viral replication in monkey CV-1 cells (Sundin and Varshavsky, 1980).

7.4. ATP dependent DNAase (ExoV) digestion of C127 pΔBR328/BPV$_I$. BamHI C1.2rc, total cellular DNA and CsCl/EtBr gradient supercoiled and open circular fraction DNA

To further investigate the high molecular weight complex of transforming DNA seen in the open circular

fraction of Fig. 7.1, and in most cellular DNAs examined so far, the enzyme ExoV was ultilised. The enzyme ExoV specifically degrades double stranded linear or single stranded DNA to oligonucleotides of average length 5.5bp, leaving supercoiled and open circular double stranded DNA molecules intact (Anai et al, 1970; Takagi et al, 1972). This enzyme has been utilised to prepare small circular DNAs from mouse thymocytes (Yamagishi et al, 1983).

The total cellular DNA extracted from the pΔBR328/BPV-1/BamHI C1.2rc cell line, and its purified supercoiled and open circular DNA fractions from Fig. 7.1, were separated undigested (-) and ExoV digested (+) on the same gel and blotted onto nitrocellulose. After probing with BPV it can be seen (Figure 7.4) that most of the material which hybridises in undigested total cellular DNA (lane 1) was degraded upon treatment with ExoV (lane 2). The pattern of circular forms remaining after digestion is identical, almost, to that seen in lane 6 (ExoV treated supercoiled fraction). This result suggests that most of the high molecular weight material present in this cell line represents linear forms of BPV-1 DNA. In the open circular fraction, lane 3 (-), a large quantity of hybridising material was detected, but this disappeared upon digestion with ExoV leaving only the open circular forms. Thus it would appear that this BPV-1 transformed cell line contained a large amount of linear viral forms which could be selectively degraded

FIGURE 7.4. ATP-dependent DNAase (Exo V) digestion of total cellular and CsCl/EtBr gradient fractionated DNAs extracted from C127 pΔBR328/BPV$_I$/BamHI Clone 2 rc.

Total cellular DNA (5μg, lanes 1 and 2) and aliquots from either the open circular fraction$^{\#}$9 (lane 3 and 4, see Fig. 7.1) or the supercoiled fraction$^{\#}$4 (lanes 5 and 6, see Fig. 7.1) were either undigested (-) or digested with an excess of Exo V (+). Samples were electrophoresed through a 0.6% agarose gel, transferred to nitrocellulose and hybridised to a nick translated probe, BPV-1.

Samples:

M; 10 copy equivalent (for 5μg) of BamHI linearised BPV-1 (7.945Kb) and HpaI linearised pAT153/BPV$_I$ (11.6Kb.) and pBR328/BPV-1/TK$_2$ (15Kb).

m; 10 copy equivalent of HpaI linearised pΔBR328/BPV$_I$ (12.5Kb).

1; Total DNA extracted from the original C127 pΔBR328/BPV$_I$/BamHI Clone 2 undigested, (-).

2; Total DNA extracted from the original C127 pΔBR328/BPV$_I$/BamHI Clone 2 Exo V digested, (+).

3; Open circular fraction #9 undigested, (-).

4; Open circular fraction #9 Exo V digested, (+).

5; Supercoiled fraction #4 undigested, (-).

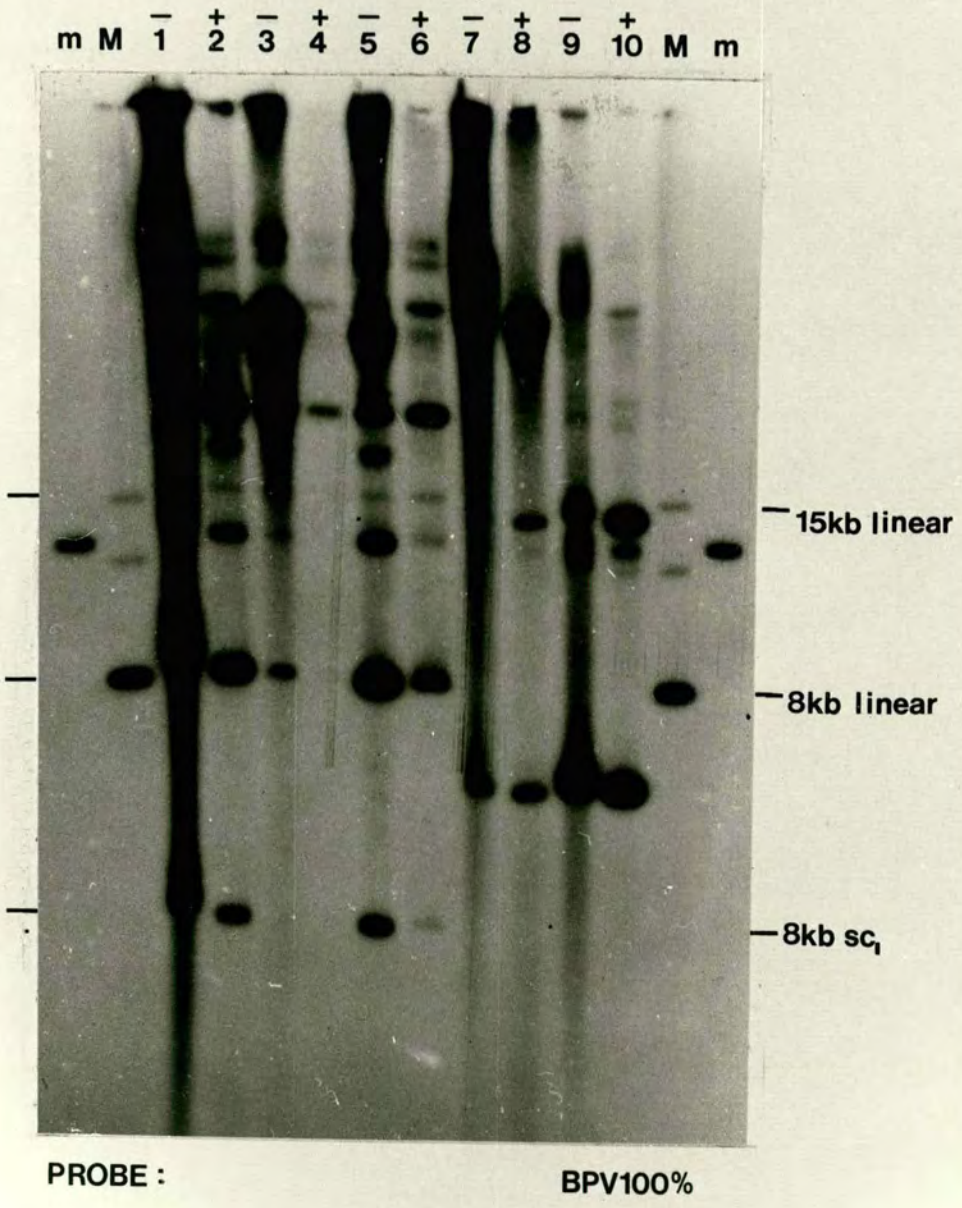
6; Supercoiled fraction #4 Exo V digested, (+).

8Kb. SC$_I$: indicates position of BPV-1 monomeric supercoiled circular form.

Lanes 7-10 : were presented and discussed in Figure 5.3.

ATP DEPENDENT DNase (ExoV) DIGESTION OF

TOTAL CELL LINE AND CsCl/EtBr GRADIENT FRACTIONATED DNA



PROBE : BPV100%

upon treatment with ExoV. Whether these forms result from breakage of large circular forms upon extraction of DNA is unknown. The fact that the majority of these linear molecules were detected in the open circular fraction after CsCl/EtBr gradient centrifugation of a carefully treated cell lysate would suggest that they were present in this state in the intact cell. Whether these linear forms were extrachromosomal or integrated is investigated in the next Section.

7.5. Zonal sucrose gradient fractionation of chromosomes prepared from the C127/pΔBR328/BPV_I/BamHI C1.2 rc cell line

Metaphase chromosomes can be separated into groups according to their size by zonal centrifugation through a 20 to 45% sucrose gradient (Padgett et al, 1977). Such centrifugation results in the separation of large chromosomes from smaller ones throughout the main body of the gradient and whole cells, nuclei and chromosome clumps are trapped in a 50% sucrose cushion. Any small extrachromosomal molecules would be retained in the uppermost fractions and, indeed, double minute chromosomes of approximately 5000kb have been separated from mouse chromosomes in this way (Tyler-Smith and Bostock, 1981).

To reveal if the linear form of BPV-1 DNA seen in

the open circular fraction of C127 pΔBR328/BPV-1/BamHI C1.2rc cell line was associated with the host cell chromosomes by integration, or represented a large extra-chromosomal form, metaphase chromosomes were prepared from this cell line and centrifuged on a 20-45% zonal sucrose gradient (see Materials and Methods Chapter 2.11). After centrifugation the gradient was fractionated into 38 x 40ml fractions and DNA isolated from each fraction. After pronase treatment and phenol/chloroform extraction an aliquot of undigested DNA from every second fraction (even numbered) was run on a 0.6% agarose gel which was subsequently blotted and hybridised with BPV-1 DNA.

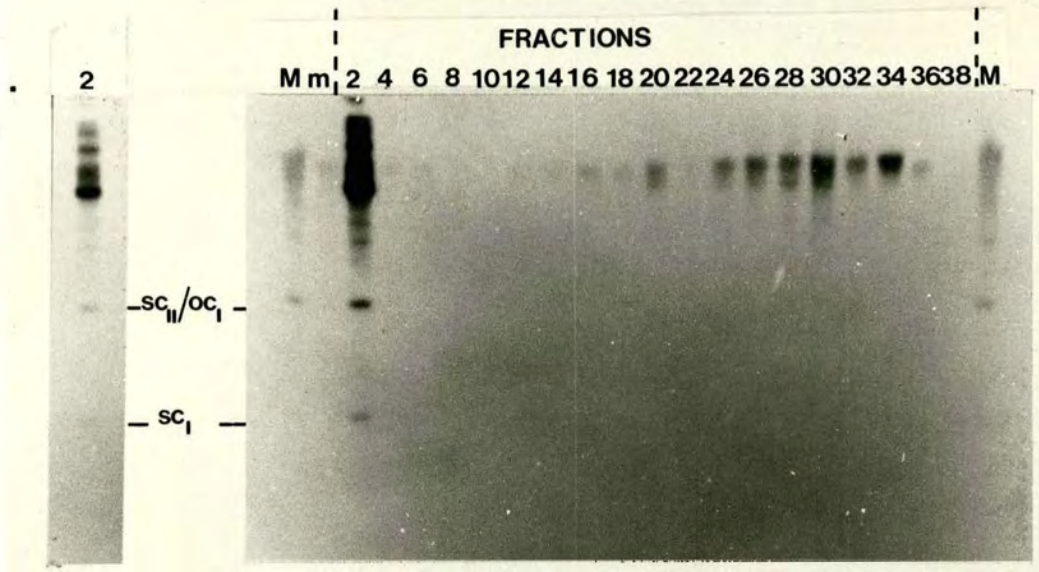
The autoradiograph in Fig. 7.5 shows that a large number of specific bands was present in non-sedimenting material contained in fraction #2. These presumably represent the extrachromosomal forms since the smaller forms comigrated with the circular forms seen in the total cellular DNA (lane M - marker). The inset in Fig. 7.5 shows a 45 minute exposure of this fraction #2 in which a very intense sharp band can be seen, with a broad diffuse band migrating just above it. It is this diffuse band which presumably represents most of the linear forms seen in total cellular DNA as well as in the open circular fractions in Fig. 7.4. This diffuse band can also be seen in the chromosomal fractions, #16 to #36. It is presumably this diffuse band (or high molecular weight smear) which represents most of the linear forms

FIGURE 7.5. Fractionation of chromosomes from the cell line C127 pΔBR328/BPV_I/BamHI Clone 2 rc by zonal sucrose gradient centrifugation.

Chromosomes prepared from the cell line were loaded onto the preformed gradient. After centrifugation the gradient was fractionated from top to bottom into 38 x 40 ml. fractions. The episomal or chromosomal DNA was extracted from each fraction. An aliquot (1/2) from every even numbered fraction was electrophoresed through a 0.65% agarose gel, transferred to nitrocellulose and hybridised to a nick translated, BPV-1.

Fractions# 2-38 are as indicated.
The inset shows a 45 min. exposure (1/2) of the hybridising material in fraction#2
M; Total cellular DNA (5μg) extracted from the original C127 pΔBR328/BPV_I/BamHI Clone 2 cell line.
m; 2.5 μg of that in M.

ZONAL SUCROSE GRADIENT FRACTIONATION OF
C127 pΔBR328/BPV/BamH1 cl2rc. CHROMOSOMES



45 min. exp.

90 min. exposure

seen in total cellular DNA as well as in the open circular fractions in Fig. 7.4. On close examination of the fractions containing the sedimenting chromosomal material the discrete, sharp band seen in fraction #2 is also visible below the diffuse linear band and on a longer exposure of this autoradiograph the smaller circular forms sc_{II}, oc_{I} and sc_{I} could be detected (data not shown). This result raises a number of possibilities. Firstly, all the BPV-1 homologous material detected in the chromosomal fractions might represent contaminating extrachromosomal material resulting from the aggregation of extrachromosomal BPV-1 chromatin molecules (linear or circular) with the chromosomes (by protein interactions). Such fast migrating extrachromosomal molecules were detected by Rosl et al (1983) when preparing BPV-1 chromatin by sucrose density centrifugation. A second possibility is that only the circular forms (discrete band) are present in the chromosomal fractions as contamination and that the diffuse linear band represents BPV-1 molecules genuinely integrated into chromosomes. Thirdly, there may be some extrachromosomal linear forms and some integrated linear forms. It is clear that even small extrachromosomal forms, which are known not to be integrated, can become associated with metaphase chromosomes and sediment with them. Thus, the mere presence of a particular form in the sedimenting fractions cannot be taken as evidence for integration. On the other hand, if the diffuse band seen in fractions #20 to #36 represents integrated, and

therefore linear, BPV-1 DNA, its proportion should be enriched in these fractions relative to the discrete extrachromosomal circular contaminating band. If it only represents contaminating extrachromosomal linear forms it should have been present in these fractions at approximately the same relative amount as the discrete contaminating circular forms.

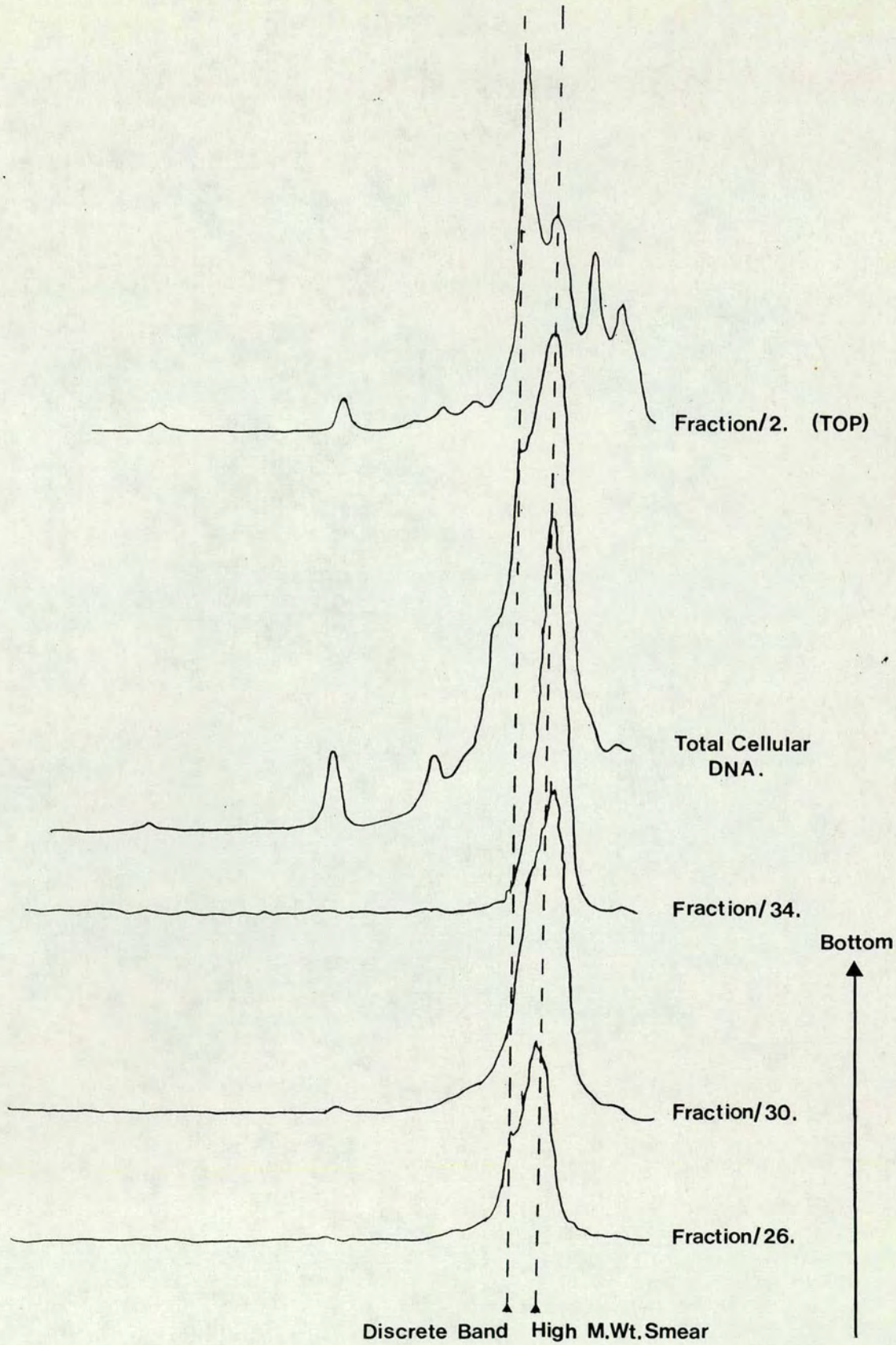
To distinguish between these possibilities the relative amounts of the diffuse high molecular weight smear and the discrete band representing a circular form below it were compared by scanning the autoradiograph with a Joyce-Loebl microdensitometer. Comparisons were made between fraction #2 containing the non-chromosomal material, the chromosomal fractions #26, #30 and #34 and total cellular DNA extracted from the same cell line. The result of these scans is presented in Fig. 7.6, where the positions of the diffuse high molecular weight smear and the discrete extrachromosomal band in each scan is indicated by the labelled broken lines. As can be seen, the main peak in total cellular DNA is formed by the diffuse high molecular smear and the discrete band is represented by a shoulder on this peak. However, when fraction #2 is examined the discrete band is the main peak and the high molecular weight smear is reduced to approximately half its relative intensity. Clearly, the fractionation procedure has enriched for the discrete band over the high molecular weight smear in fraction 2 which contains the non-sedimenting extrachromosomal

FIGURE 7.6.

Joyce-Loebl Microdensitometer scan comparing the relative intensities of the high molecular weight smear and the discrete extrachromosomal band in Fractions #2 (top fraction), Fractions #26, #30 and #34 (chromosomal fractions) and total DNA extracted from the original cell line. Scans were carried out on the autoradiograph shown in Fig. 7.5 and on shorter exposures.

The positioning of these two components is indicated by the broken lines.

Top and bottom refer to the position of the fractions in the gradient.


Fraction/2. (TOP)
Total Cellular DNA.
Fraction/34.
Bottom
Fraction/30.
Fraction/26.
Discrete Band
High M.Wt. Smear

material. On examining the scans of the three chromosomal fractions, however, the high molecular weight smear is found to predominate and in fraction #34 none of the contaminating discrete extrachromosomal band was detected on the scan. Therefore comparison of the relative amounts of these two bands in different fractions and in total cellular DNA reveals that the material represented by the diffuse high molecular weight smear, which hybridises to BPV-1, is enriched for in the chromosomal fractions and therefore must, in a rather tight way, be associated with a host cell chromosome or chromosomes. Such a tight association would be consistent with some copies of BPV-1 integrating into host chromosomal material. However, since some of this high molecular weight material was also detected in fraction #2, it is possible that a proportion of this material may represent large extrachromosomal linear forms.

Many attempts to digest with restriction enzymes the DNA from fractions #2, #28 and #30 have failed and thus a junction fragment resulting from digestion on either side of the viral/host DNA integration site has not as yet been revealed. The use of <u>in situ</u> hybridisation of BPV-1 DNA to chromosomes prepared from this cell line should confirm the presence or absence of chromosomally integrated BPV-1 DNA, and such an analysis is currently in progress.

7.6. Discussion

In Chapter 3 it was confirmed that the BPV-1 isolate used in this study behaves identically to that of Sarver *et al* (1981) when introduced into C127 cells under similar culture and transformation conditions.

The results presented in this Chapter have taken a close look at the DNA of one particular cell line which was derived by the selection of a focus resulting from the introduction, by precipitation, of BamHI cleaved pΔBR328/BPV-1. They have revealed the persistence of vector sequences as integrated or high molecular weight forms in these cell lines. Furthermore, analysis by CsCl-EtBr gradient fractionation of a cell lysate made from this cell line has revealed the presence of large concatemeric supercoiled structures of up to 48kb, and possibly 80kb, in size,which must therefore consist of 6, and possibly up to 10, BPV-1 genomes linked in one circular form.

The possibility that BPV-1 sequences were integrated into a host chromosome in this cell line was also investigated by revealing upon ExoV digestion that a major proportion of the BPV-1 DNA sequences were present as linear molecules. These linear forms may have been derived from broken large circular forms or chromosomally integrated viral sequences. Fractionation of chromosomes prepared from the cell line upon a zonal sucrose gradient

further suggested the integration of these BPV-1 linear forms into a host chromosome or chromosomes. Whether such an integration event is the consequence of the joining of a proportion of the viral molecules with co-transfected pΔBR328 sequences, or is a natural event in the replication of BPV-1 in mammalian cells, is not as yet known.

It is interesting to note that the work of others also suggests that some BPV-1 sequences may be integrated into host chromosome in transformed cell lines:- The data of Breitburd et al (1981) suggests that in one of their tumorigenic hamster cell lines, HT3, which was derived from a BPV-1 induced tumor, some viral DNA sequences had integrated into a host chromosome, since additional unexpected bands were detected following both BamHI and EcoRI digestion. Such bands could represent viral DNA host chromosome junctions at the site of integration.

Recently, Sauer et al (1984) have demonstrated the ability of xanthate compounds to act as antiviral agents. They demonstrate that the xanthate compound D609 could reduce the yield of Herpes Simplex Virus (HSV) types 1 and 2 by 50% when applied to infected cells in culture. The compound did not effect the proliferation of non-virally infected human, primate, rodent or murine cells. To further investigate the antiviral action of this compound, its effect on the

replication of SV40 in CV-1 cells and BPV-1 in HEF (Hamster Embryo Fibroblasts) cells was examined by Southern and Northern blotting. This analysis revealed that treatment with D609 abolished both SV40 and BPV-1 transcription and replication. Even more striking was the fact that the D609 treated BPV-1 transformed HEF cells reverted to a non-transformed phenotype since they displayed a flat morphology and regained contact inhibition. Although DNA from these "cured" HEF cell lines was found to contain no monomeric supercoiled or open circular forms of BPV-1, a slow migrating complex was still detected as a single diffuse band. Although not referred to in the paper, this suggests that this complex may be integrated into a host chromosome since the xanthate treatment has removed all replicating monomeric extrachromosomal forms. If extrachromosomal, a high molecular weight extrachromosomal element would also have been expected to be diluted out upon proliferation (three passages) of these cells in the presence of D609. Unfortunately, no futher analysis of this 'cured' cell line was performed. Both of these reports involve the replication of BPV-1 in Hamster cell lines so the occurrence of similar events in mouse C127 cell lines transformed with BPV-1 has not as yet been reported.

Turek et al (1982), have found that mouse L-cell interferon has an effect similar to xanthates, on C127 cell lines transformed with BPV-1 virions. The amount of BPV-1 DNA remaining after 60 generations of growth in

medium containing interferon was reduced by up to 8-fold. Flat revertants (non-transformed) cell lines were isolated from interferon treated cell lines which were susceptable to BPV-1 retransformation. Thus, interferon could also be used to investigate the status of the high molecular linear forms in transformed cells. It is possible that integrated sequences would not be lost upon treatment with interferon, as observed in the xanthate treated hamster cell lines.

The work presented raises the possibility that BPV-1 integrates into a host chromosome in a transformed C127 mouse cell line. This has important implications for the use of BPV- 1 as a vector for investigation of the function, expression or otherwise, of sequences on a supposedly extrachromosomal vector. Clearly, the possibility exists that viral DNA can integrate and needs to be investigated more closely.

CHAPTER 8

DISCUSSION AND CONCLUSIONS

In Chapter 1.9 a set of properties were identified, which should be incorporated into a truly extra-chromosomal replicating mammalian cell vector. Attempts to construct such a vector were disappointing, since it was found that many of the constructs underwent deletion events at a high frequency. These were subsequently shown to effect mainly the plasmid and/or selectable marker sequences when selection for the BPV-1 transformation function was applied. In many cases, selection for a dominant selectable marker carried by the vector led to the incorporation of the vector into a high molecular weight complex with no detectable intact extrachromosomal monomeric circular forms present in those cell lines.

The presence of just this high molecular weight complex is reminiscent of the transgenome structures formed in the transformation experiments involving the HSV-TK gene and cotransferred sequences discussed in the Chapter 1 (Sections 1 to 3). Indeed it is reasonable to expect that selection for a dominant selectable marker, without selection for BPV-1 replication (as in Ltap207 cells), would result in the aggregation of the transferred plasmid sequences into a transgenome which eventually integrates into a host chromosome. However, the fact that this high molecular weight material was detected in cell lines transformed by precipitation or injection methods with BPV-1 DNA only (Fig. 3.1. A and D),

and that it has been detected in DNA extracted from cell lines transformed with BPV-1 virions with subsequent selection for focus formation (Law et al, 1981, Lancaster, 1981), suggests that this material results from the replication of viral DNA in C127 cells.

The structure of this high molecular weight complex, in a cell line transformed with BPV-1, was investigated in Chapter 7. It was found that this material consisted of two components: one component was composed of a discrete sized concatemeric (oligomeric multimers) open circular and supercoiled extrachromosomal circular forms of up to approximately 80kb in size. The second component consisted of material which was detected as a heterogeneous diffuse smear on agarose gels and which comigrated with the top of the excluded high molecular weight DNA. It must therefore, represent linear DNA molecules of size >30kb. Some of this material may have represented extrachromosomal linear forms which resulted from the breakage of the large circular forms. Fractionation of chromosomes from a cell line revealed that most of this high molecular weight linear material was associated with chromosomes and was probably integrated. Whether or not these integrated forms resulted from the association of BPV-1 with plasmid sequences is not known.

BPV-1 transformed C127 cell lines contain a complex mixture of different forms of the viral DNA. Some of

these forms may be a direct consequence of the replication of BPV-1 in a non-productive cell, in which all the functions and products necessary for viral DNA replication are available. The products of replication cannot, however, be packaged into virions, and, therefore, remain in the cells. The products of replication may include high molecular weight forms which could conceivably represent substrates for packaging. Alternatively, the high molecular weight forms may result from intermolecular homologous or non-homologous recombination between the many monomeric copies present in the cell. Such events have been shown to occur between DNA molecules microinjected into mammalian cells nuclei (Folger et al, 1982). Integration of viral DNA into a host chromosome may result from the formation of defective viral molecules upon replication in the mammalian cell. One could envisage a viral molecule integrating into the host genome after it had lost its origin of replication due to mutation. Indeed, there are many reports of the high mutation frequency encountered by DNA transfected into cultured mammalian cells (Calos et al, 1983; Razzaque et al, 1983, and Wake et al, 1984).

The possibility that BPV-1 DNA alone can integrate into the host genome, without there being any influence of linked prokaryotic or eukaryotic sequences, has important implications for the use of these vectors. It is hoped that in situ hybridisation of labelled BPV-1

DNA to metaphase chromosomes prepared from BPV-1 transformed cell lines and techniques such as pulse field electrophoresis (Van der Ploeg et al, 1984; Carle and Olson, 1984) of the high molecular weight forms, will assist in the further resolution of the structure of these BPV-1 complexes in transformed cells. Time-course experiments in which the fate of transfected DNA in a cell population can be followed may be useful in determining the processes involved in the formation of all these complex DNA forms.

The vectors constructed in the course of this work were based on the complete genome which was inserted into the plasmids pBR328, pAT153 or deletion derivatives of them. None of these vectors were found to replicate in the desired manner, that is as intact, unrearranged extrachromosomal, monomeric circular forms. When the vectors were modified as far as possible to resemble those vectors which have been reported to replicate in the desired manner, such as pML2d/BPV-1, examination of the resulting cell line DNA's showed the replicating forms to be deleted in size. The size of these deleted forms (6-9kb) was comparable to the sizes of the BPV69%T fragment (5.44kb) or the BPV-1 complete genome (7.945kb). Both of these have been shown to replicate as monomeric extrachromosomal forms in transformed C127 cells, although in order to do so the BPV69%T fragment may have to acquire additional sequences (Law et al, 1981; DiMaio et al, 1982). In some cases a selectable marker

(HSV-TK PuvII, or SV_2Neo) was inserted into the vector, but cell lines resulting from selection for the marker contained only high molecular weight forms. In some focus selected C127 cell lines transformed with vectors such as pAT153/BPV_I or pBR328/BPV_I, only high molecular weight complexed DNA could be detected. Attempts to purify supercoiled forms from one of these cell lines failed (data not shown) and since all the hybridising material was in the open circular/linear fraction it is likely that the vector was present as a large transgenome type structure (see Chapter 1) which may or may not have been integrated into a host cell chromosome.

Comparison of the BPV-1 genome with those of SV40 and polyoma viruses indicated a number of similarities between the relative positions of the viral origins of replication, the enhancer sequences and the transcriptional promoters. During construction of all the vectors the bacterial plasmid sequences had been inserted between the BPV-1 enhancer and its promoter and origin of replication (PMS-1), so that in one direction around the circular genome they are separated by an additional 3.3 to 4.9kb (depending on the plasmid used). This arrangement results in the inclusion of a known bacterial substitute promoter ('i' - see Fig. 2.6 - Wasylyk et al, 1983) between the viral enhancer and promoter region, which may result in a considerable reduction of transcription from the viral promoter as has

been found by Wasylyk et al (1983 and 1984) in SV40 constructs. In order to test the above hypothesis, the BPV-1 enhancer was duplicated so as to be either directly adjacent to the promoter region, as in the normal circular BPV-1 genome, or adjacent to the original enhancer and still separated by an extra 3.3kb of plasmid from the promoter region. These manipulations resulted in the formation of vectors, a proportion of which were found to be maintained (as far as is discernable) as intact, unrearranged, extrachromosomal monomeric forms in some transformed cell lines. However, they did undergo deletion events in other similarly transformed cell lines and were therefore not 100% stable (see Fig. 4.7). The duplication of the viral enhancer, clearly had some effect on the stability of these plasmid-BPV-1 hybrid vectors, but the fact that all three of the enhancer duplication vectors showed some stability, regardless of the position of the duplication was surprising and is difficult to explain. It is conceivable that the effect of having two enhancers was additive, allowing transcription to be promoted over longer distances and even across a substitute promoter which is proximal to the viral promoter region.

In an attempt to test the transformation conditions used for all the experiments described here, BPV-1 vectors from other laboratories were obtained and introduced into C127 cells. The results were variable. One vector, pBrd/BPV-β_1 replicated as an intact

extrachromosomal circular molecule, as reported by DiMaio et al (1982), when introduced into C127 cells by either the precipitation or injection method. $pCGBPV_{9\Delta B5}$ only functioned in the reported manner (Matthias et al, 1983) when introduced by microinjection and not after calcium phosphate precipitation, contrary to the results of P. Matthias (pers. comm.). The vector pML2d/BPV-1 (Sarver et al, 1982), on which many of the vectors constructed in this thesis were based, was found not to replicate as an intact episome, but, like many of the vectors constructed, deleted to a size clearly similar to that of BPV-1 itself. These results are, to say the least, confusing, in that one vector functions in complete agreement with the published work whereas a second vector introduced under the same conditions does not do so. These conflicting results could, as discussed in Chapter 5, result from variable factors such as the quality of a particular DNA precipitate, the use of carrier DNA-free precipitates or variability in the culture conditions. These possibilities remain to be tested. However, one explanation for the positive results obtained with the pBrd/BPV-β_1 vector would be if the human β-globin fragment, which is inserted adjacent to the BPV-1 promoter region (see Fig. 2.7) contains an enhancer type sequence which retains this activity to some extent in mouse C127 cells. Such enhancement would allow the replication of the vector without deletion of the sequences intervening between the viral enhancer and the viral promoter. The recent

finding by DeVilliers et al (1984) is relevant here. They showed that the presence of an enhancer sequence is an actual requirement for replication of polyoma virus, even in cells which produce all the required viral products for replication in trans. This stresses that the BPV-1 genome may also require a functional enhancer, not only for transcription, but perhaps to play some structural role in viral replication.

The fact that the pEN-DUP-D and B forms of the enhancer duplication vectors were recoverable into competent E.coli is encouraging. The recovery of these plasmids will allow them to be mapped in detail and, therefore, the determination of whether they contain any minor alterations in their structure which might be responsible for their maintenance as 'intact' monomeric circular forms in transformed cells. The recovered vectors are currently being tested for their continued stability when reintroduced into C127 cells in a second round of transformation.

The recovery of deleted forms (17.1, 17.4 and 17.5) of the pΔBR328/BPV$_I$ vector (see Table 6.1, sample #17 and Fig. 6.5) raises the possibility that these have retained the minimal BPV-1 sequences needed for extra-chromosomal replication in a C127 cell in which all other products required in trans are provided by a co-replicating complete BPV-1 genome. Secondary transformation of non-transformed C127 cells and a

transformed C127 cell line containing BPV-1 should reveal whether these deletants are capable of independent replication or whether they require BPV-1 product(s) to be provided in *trans*. Fine mapping and sequencing of these mini plasmids would perhaps enable them to be used as the basis of a BPV-1 vector carrying a selectable marker similar to those constructed by Lusky and Botchan (1984) and Waldeck *et al* (1984). Given their ability to replicate it will be of interest to see if these mini-plasmids retain the viral enhancer sequence.

The aim of using such extrachromosomal vectors as a means of introducing libraries of cloned genomic DNA into mammalian cells, and easily identifying and isolating the sequences encoding a particular phenotype carried by a member of that library has not, as yet, been realised. The causes of the integration of vectors when particular sequences are inserted (U1snRNA in $pCGBPV_{9\Delta B5}$; Human HLA in pBRd BPV-β_1 DiMaio *et al*, 1984 - see Chapter 5.8) has not yet been investigated. It is possible that such problems are mainly due to the relative positioning of the inserted sequences and functional regions of the vector. Perhaps manipulations of the vector sequences, such as duplication of the enhancer, and deletion of certain bacterial sequences, will eventually contribute to a fully functional BPV-1 shuttle type vector, which can be easily selected for and which replicates extra-chromosomally as an unrearranged and unintegrated form in both *E.coli* and mammalian cells. However, the problem

of possible integration of the viral DNA into the mammalian host genome remains, and until this can be solved the use of BPV-1 based vectors will be limited.

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